

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
5 July 2001 (05.07.2001)

PCT

(10) International Publication Number
WO 01/48183 A2

- (51) International Patent Classification⁷: **C12N 15/00**
- (21) International Application Number: PCT/EP00/13149
- (22) International Filing Date:
22 December 2000 (22.12.2000)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:
9930691.2 24 December 1999 (24.12.1999) GB
- (71) Applicant (*for all designated States except US*): **DEV-GEN NV** [BE/BE]; Technologiepark 9, B-9052 Zwijnaarde (BE).
- (72) Inventors; and
- (75) Inventors/Applicants (*for US only*): **PLAETINCK, Geert** [BE/BE]; Pontstraat 16, B-9820 Merelbeke (BE). **MORTIER, Katherine** [BE/BE]; Paddenhoek 20, B-9830 St.-Martens Latem (BE). **LISSENS, Ann** [BE/BE]; Tiensesteenweg 137, B-3010 Kessel-Lo (BE). **BOGAERT, Thierry** [BE/BE]; Wolvendreef 26g, B-8500 Kortrijk (BE).
- (74) Agent: **BAYLISS, Geoffrey, Cyril**; Boulton Wade Tennant, Verulam Gardens, 70 Gray's Inn Road, London WC1X 8BT (GB).
- (81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.
- (84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).
- Published:**
— Without international search report and to be republished upon receipt of that report.
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

(54) Title: IMPROVEMENTS RELATING TO DOUBLE-STRANDED RNA INHIBITION

(57) Abstract: There are described ways of improving the efficiency of double stranded RNA inhibition as a method of inhibiting gene expression in nematode worms such as *C. elegans*. In particular, the invention relates to the finding that changes in the genetic background of *C. elegans* result in increased sensitivity to double-stranded RNA inhibition.



WO 01/48183 A2

- 1 -

IMPROVEMENTS RELATING TO DOUBLE-STRANDED RNA
INHIBITION

5 The present invention is concerned with ways of
improving the efficiency of double stranded RNA
inhibition as a method of inhibiting gene expression
in nematode worms such as *C. elegans*. In particular,
the invention relates to the finding that the
susceptibility of nematode worms such as *C. elegans* to
10 double stranded RNA inhibition is affected by changes
in the genetic background of the worms.

It has recently been described in Nature Vol 391,
pp.806-811, February 98, that introducing double
stranded RNA into a cell results in potent and
15 specific interference with expression of endogenous
genes in the cell, which interference is substantially
more effective than providing either RNA strand
individually as proposed in antisense technology. This
specific reduction of the activity
20 of the gene was also found to occur in the nematode
worm *Caenorhabditis elegans* (*C. elegans*) when the RNA
was introduced into the genome or body cavity of the
worm.

The present inventors have utilized the double
25 stranded RNA inhibition technique and applied it
further to devise novel and inventive methods of (i)
assigning functions to genes or DNA fragments which
have been sequenced in various projects, such as, for
example, the human genome project and which have yet
30 to be accorded a particular function, and (ii)
identifying DNA responsible for conferring a
particular phenotype. Such methods are described in
the applicant's co-pending application number WO
00/01846. Processes for introducing RNA into a living
35 cell, either *in vivo* or *ex vivo*, in order to inhibit
expression of a target gene in that cell are

additionally described in WO 99/32619.

Several different experimental approaches can be used to introduce double-stranded RNA into nematode worms in order to achieve RNA interference *in vivo*.

5 One of the most straightforward approaches is simple injection of double-stranded RNA into a body cavity. A more elegant solution is to feed the nematodes on food organisms, generally bacteria, which express a double stranded RNA of the appropriate sequence,
10 corresponding to a region of the target gene.

The present inventors have now determined that the phenomenon of RNA interference in nematodes following ingestion of food organisms capable of expressing double-stranded RNA is dependent both on
15 the nature of the food organism and on the genetic background of the nematodes themselves. These findings may be exploited to provided improved methods of double-stranded RNA inhibition.

Therefore, according to a first aspect of the
20 present invention there is provided a method of inhibiting expression of a target gene in a nematode worm comprising feeding to said nematode worm a food organism which is capable of producing a double-stranded RNA structure having a nucleotide sequence
25 substantially identical to a portion of said target gene following ingestion of the food organism by the nematode, wherein the nematode has a non wild-type genetic background selected to provide increased sensitivity to RNA interference as compared to wild
30 type.

Caenorhabditis elegans is the preferred nematode worm for use in the method of the invention although the method could be carried out with other nematodes and in particular with other microscopic nematodes,
35 preferably microscopic nematodes belonging to the genus *Caenorhabditis*. As used herein the term "microscopic" nematode encompasses nematodes of

approximately the same size as *C. elegans*, being of the order 1mm long in the adult stage. Microscopic nematodes of this approximate size can easily be grown in the wells of a multi-well plate of the type
5 generally used in the art to perform mid- to high-throughput screening.

It is an essential feature of this aspect of the invention that the nematode has a non wild-type genetic background which confers greater sensitivity
10 to RNA interference phenomena (abbreviated herein to RNAi) as compared to the equivalent wild type nematodes. As illustrated in the accompanying examples, introduction of double-stranded RNA (abbreviated herein to dsRNA) into a non wild-type
15 strain according to the invention results in greater inhibition of expression of the target gene. Depending on the nature of the target gene, this greater level of inhibition may be detectable at the phenotypic level as a more pronounced phenotype.

20 The nematode having non wild-type genetic background may, advantageously, be a mutant strain. Mutations which have the effect of increasing susceptibility of the nematode to RNAi may, for example, affect the stability of dsRNA or the kinetics
25 of dsRNA turnover within cells of the worm or the rate of uptake of dsRNA synthesised by a food organism. Suitable mutant strains include mutant strains exhibiting knock-out or loss-of-function mutations in one or more genes encoding proteins involved in RNA
30 synthesis, RNA degradation or the regulation of these processes.

In one preferred embodiment, the nematode is a mutant strain, more preferably a mutant *C. elegans*, which exhibits reduced activity of one or more
35 nucleases compared to wild-type. Suitable strains include mutant strains exhibiting knock-out or loss-of-function mutations in one or more genes encoding

nucleases, such as RNases. A particularly preferred example is the *nuc-1* strain. This mutant *C. elegans* strain is known *per se* in the art.

5 In a second preferred embodiment, the nematode is a mutant strain, more preferably a mutant *C. elegans*, which exhibits increased gut uptake compared to wild-type. Particularly preferred examples of such strains are the so-called *C. elegans* gun mutants described herein. In a still further embodiment, the nematode
10 may be a transgenic worm comprising one or more transgenes which increase gut uptake relative to wild-type.

The term "increased gut uptake" as used herein is taken to mean increased uptake of foreign particles
15 from the gut lumen and may encompass both increased gut permeability and increased gut molecular transport compared to wild-type *C. elegans*.

C. elegans feeds by taking in liquid containing its food (e.g. bacteria). It then spits out the
20 liquid, crushes the food particles and internalises them into the gut lumen. This process is performed by the muscles of the pharynx. The process of taking up liquid and subsequently spitting it out is called pharyngeal pumping. Once the food particles have been
25 internalised via pharyngeal pumping their contents must cross the gut itself in order to reach target sites in the worm. There are multiple factors which effect the uptake of compounds from the gut lumen to the surrounding tissues. These include the action of
30 multi-drug resistance proteins, multi-drug resistance related proteins and the P450 cytochromes as well as other enzymes and mechanisms available for transport of molecules through the gut wall.

C. elegans mutants which exhibit increased uptake
35 of foreign molecules through the gut may be obtained from the *C. elegans* mutant collection at the C.

C. elegans Genetic Center, University of Minnesota, St Paul, Minnesota, or may be generated by standard methods. Such methods are described by Anderson in Methods in Cell Biology, Vol 48, "*C. elegans*: Modern biological analysis of an organism" Pages 31 to 58. Several selection rounds of the PCR technique can be performed to select a mutant worm with a deletion in a desired gene. Alternatively, a population of worms could be subjected to random mutagenesis and worms exhibiting the desired characteristic of increased gut uptake selected using a phenotypic screen, such as the dye uptake method described herein.

As an alternative to mutation, transgenic worms may be generated with the appropriate characteristics. Methods of preparing transgenic worms are well known in the art and are particularly described by Craig Mello and Andrew Fire, Methods in Cell Biology, Vol 48, Ed. H.F. Epstein and D.C. Shakes, Academic Press, pages 452-480.

Worms exhibiting the desired characteristics of increased gut uptake can be identified using a test devised by the inventors based on uptake of a marker precursor molecule which is cleaved by the action of enzymes present in the gut lumen to generate a marker molecule which produces a detectable signal, such as fluorescence. A suitable marker precursor molecule is the fluorescent dye precursor BCECF-AM available from Molecular Probes (Europe BV), Netherlands. This dye only becomes fluorescent when cleaved by esterases and maintained at a pH above 6. The pH of the gut lumen is usually 5 or below. Thus, any BCECF-AM taken up through the pharynx into the gut lumen is not fluorescent until cleaved and the cleaved portion has entered the cells surrounding the lumen which are at a higher pH. Thus, this dye is able to quickly identify mutant or otherwise modified worms which have increased gut transport or permeability. There is a

gradual increase in fluorescence in the tissues surrounding the gut while the gut lumen remains dark. The fluorescence can be detected at an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

Specific examples of gun mutant strains isolated using this procedure which may be used in the method of the invention are strains bg77, bg84, bg85 and bg86, although it is to be understood that the invention is in no way limited to the use of these specific strains. The *C. elegans* gun mutant strain bg85 was deposited on 23 December 1999 at the BCCM/LMG culture collection, Laboratorium Voor Microbiologie, Universiteit Gent, K. L. Ledeganckstraat 35, B-9000, Gent, Belgium under accession number LMBP 5334CB. The phrase "the bg85 mutation" as used herein refers to the specific mutation(s) present in the bg85 strain which is/are responsible for conferring the gun phenotype.

It is also within the scope of the invention to use a non wild-type nematode strain, preferable a *C. elegans* strain, having multiple mutations which affect sensitivity to RNAi. A preferred type of multiple mutant is one having at least one mutation which results in reduced nuclease activity compared to wild type and at least one mutation which results in increased gut uptake compared to wild type. An example of such a mutant is a *C. elegans* strain having the *nuc-1* mutation and at least one further gun mutation. As exemplified herein, double mutants having the *nuc-1* mutation and a gun mutation exhibit enhanced sensitivity to RNAi as compared to either *nuc-1* or gun single mutants.

For the avoidance of doubt, where particular characteristics or properties of nematode worms are described herein by relative terms such as "enhanced"

or "increased" or "decreased" this should be taken to mean enhanced, increased or decreased relative to wild-type nematodes. In the case of *C. elegans*, wild-type is defined as the N2 Bristol strain which is well known to workers in the *C. elegans* field and has been extremely well characterised (see Methods in Cell Biology, Volume 48, *Caenorhabditis elegans: Modern biological analysis of an organism*, ed. by Henry F. Epstein and Diane C. Shakes, 1995 Academic Press; The nematode *Caenorhabditis elegans*, ed. by William Wood and the community of *C. elegans* researchers., 1988, Cold Spring Harbor Laboratory Press; *C. elegans* II, ed. by Donald L. Riddle, Thomas Blumenthal, Barbara J. Meyer and James R. Priess, 1997, Cold Spring Harbor Laboratory Press). The N2 strain can be obtained from the *C. elegans* Genetic Center, University of Minnesota, St Paul, Minnesota, USA.

The food organism for use in the above aspect of the invention is preferably a bacterium such as, for example, a strain of *E.coli*. It will, however, be appreciated that any other type of food organism on which nematodes feed and which is capable of producing dsRNA could be used. The food organism may be genetically modified to express a double-stranded RNA of the appropriate sequence, as will be understood with reference to the examples included herein. One convenient way in which this may be achieved in a bacterial food organism is by transforming the bacterium with a vector comprising a promoter or promoters positioned to drive transcription of a DNA sequence to RNA capable of forming a double-stranded structure. Examples of such vectors will be further described below.

The actual step of feeding the food organism to the nematode may be carried out according to procedures known in the art, see WO 00/01846.

Typically the feeding of the food organisms to the nematodes is performed on standard agar plates commonly used for culturing *C. elegans* in the laboratory. However, the step of feeding the food
5 organism to the nematodes may also be carried out in liquid culture, for example in the wells of 96-well microtitre assay plates.

The inventors have further observed that variations in the food organism can result in enhanced
10 *in vivo* RNAi when the food organism is ingested by a nematode worm.

Accordingly, in a further aspect the invention provides a method of inhibiting expression of a target gene in a nematode worm comprising feeding to said
15 nematode worm a food organism capable of producing a double-stranded RNA structure having a nucleotide sequence substantially identical to a portion of said target gene following ingestion of the food organism by the nematode, wherein the food organism carries a
20 modification selected to provide increased expression or persistence of the double-stranded RNA compared to a food organism which does not carry the modification.

The modification present in the food organism can be any modification which results in increased
25 expression of the dsRNA or in increased persistence of the dsRNA. Suitable modifications might include mutations within the bacterial chromosome which affect RNA stability and/or degradation or mutations which have a direct effect on the rate of transcription. In
30 a preferred embodiment, the food organism is an RNase III minus *E. coli* strain, or any other RNase negative strain.

According to a still further aspect of the invention there is provided a method of inhibiting
35 expression of a target gene in a nematode worm comprising introduction of a DNA capable of producing a double-stranded RNA structure having a nucleotide

13 sequence substantially identical to a portion of said target gene in said nematode, wherein the nematode is one which exhibits increased gut uptake compared to wild type.

5 In addition to exhibiting increased sensitivity to RNAi following feeding with food organisms capable of expressing a dsRNA, nematodes which exhibit increase gut uptake as described herein also show increased uptake of DNA molecules capable of producing
10 double-stranded RNA structures following ingestion into a nematode.

In a preferred embodiment, the DNA is in the form of a vector comprising a promoter or promoters orientated to relative to a sequence of DNA such that
15 they are capable of driving transcription of the said DNA to make RNA capable of forming a double-stranded structure upon binding of an appropriate RNA polymerase to the promoter or promoters.

Several different arrangements of promoters may
20 be used in such a vector. In a first arrangement a DNA fragment corresponding to a region of the target gene is flanked by two opposable polymerase-specific promoters which are preferably identical. Transcription from the opposable promoters produces
25 two complementary RNA strands which can anneal to form an RNA duplex. The plasmid pGN1 described herein is an example of a vector comprising two opposable T7 promoters flanking a multiple cloning site for insertion of a DNA fragment of the appropriate
30 sequence, corresponding to a region of a target gene. pGN8 is an example of a vector derived from pGN1 containing a fragment of the *C. elegans unc-22* gene. In an alternative arrangement, DNA fragments corresponding to a region of the target gene may be
35 placed both in the sense and the antisense orientation downstream of a single promoter. In this case, the sense/antisense fragments are co-transcribed to

generate a single RNA strand which is self-complementary and can therefore form an RNA duplex.

In both of the above arrangements, the polymerase-specific T3, T7 and SP6 promoters, all of which are well known in the art, are useful for driving transcription of the RNA. Expression from these promoters is dependent on expression of the cognate polymerase. Advantageously, the nematode itself may be adapted to express the appropriate polymerase. Expression of the polymerase may be general and constitutive, but could also be regulated under a tissue-specific promoter, an inducible promoter, a temporally regulated promoter or a promoter having a combination of such characteristics. Transgenic *C. elegans* strains harboring a transgene encoding the desired polymerase under the control of an appropriately-regulated promoter can be constructed according to methods known *per se* in the art and described, for example, by Craig Mello and Andrew Fire in *Methods in Cell Biology*, Vol 48, Ed. H. F. Epstein and D. C. Shakes, Academic Press, pp 452-480.

The advantage of adapting the nematode to express the required polymerase is that it is possible to control inhibition of expression of the target gene in a tissue-specific and/or temporally specific manner by placing expression of the polymerase under the control of an appropriately regulated promoter.

Introduction of DNA into nematodes in accordance with the method of the invention can be achieved using a variety of techniques, for example by direct injection into a body cavity or by soaking the worms in a solution containing the DNA. If the DNA is in the form of a vector as described herein, e.g. a plasmid harboring a cloned DNA fragment between two flanking T7 promoters, then dsRNA corresponding to this DNA fragment will be formed in the nematode resulting in down regulation of the corresponding gene. The

introduced DNA can form an extrachromosomal array, which array might result in a more catalytic knock-out or reduction of function phenotype. The DNA might also become integrated into the genome of the nematode, resulting in the same catalytic knock out or reduction of function phenotype, but which is stably transmittable.

In each aspect of the invention, the double-stranded RNA structure may be formed by two separate complementary RNA strands or a single self-complementary strand, as described above. Inhibition of target gene expression is sequence-specific in that only nucleotide sequences corresponding to the duplex region of the dsRNA structure are targeted for inhibition.

It is preferred to use dsRNA comprising a nucleotide sequence identical to a portion of the target gene, although RNA sequences with minor variations such as insertions, deletions and single base substitutions may also be used and are effective for inhibition. It will be readily apparent that 100% sequence identity between the dsRNA and a portion of the target gene is not absolutely required for inhibition and the phrase "substantially identical" as used herein is to be interpreted accordingly. Generally sequences which are substantially identical will share at least 90%, preferably at least 95% and more preferably at least 98% nucleic acid sequence identity. Sequence identity may be conveniently calculated based on an optimal alignment, for example using the BLAST program accessible at WWW.ncbi.nlm.nih.gov.

The invention will be further understood with reference to the following non-limiting Examples, together with the accompanying Figures in which:

Figure 1 is a plasmid map of the vector pGN1

containing opposable T7 promoters flanking a multiple cloning site and an ampicillin resistance marker.

5 Figure 2 is a plasmid map of the vector pGN8 (a genomic fragment of the *C. elegans unc-22* gene cloned in pGN1).

10 Figure 3 is a plasmid map of the vector pGN29 containing two T7 promoters and two T7 terminators flanking *Bst*XI sites. This vector permits cloning of DNA fragments linked to *Bst*XI adaptors.

15 Figure 4 is a plasmid map of the vector pGN39 containing two T7 promoters and two T7 terminators flanking attR recombination sites (based on the Gateway™ cloning system of Life Technologies, Inc).

20 Figure 5 is a plasmid map of the vector pGX22 (a fragment of the *C. elegans* gene C04H5.6 cloned in pGN29).

25 Figure 6 is a plasmid map of the vector pGX52 (a fragment of the *C. elegans* gene K11D9.2b cloned in pGN29).

Figure 7 is a plasmid map of the vector pGX104 (a fragment of the *C. elegans* gene Y57G11C.15 cloned in pGN29).

30 Figure 8 is a plasmid map of the vector pGZ8 (a fragment of the *C. elegans* gene T25G3.2 cloned in pGN39).

35 Figure 9 shows the results of an RNAi experiment in which wild-type (N2) or *nuc-1* strain *C. elegans* in liquid culture were fed with *E. coli* containing the

plasmid pGX22.

Figure 10 shows the results of an RNAi experiment in which wild-type (N2) or *nuc-1* strain *C. elegans* in
5 liquid culture were fed with *E. coli* containing the plasmid pGX52.

Figure 11 shows the results of an RNAi experiment in which wild-type (N2) or *nuc-1* strain *C. elegans* in
10 liquid culture were fed with *E. coli* containing the plasmid pGXGZ8.

Figure 12 shows the results of an RNAi experiment in which wild-type (N2) or *nuc-1* strain *C. elegans* in
15 liquid culture were fed with *E. coli* containing the plasmid pGX104

20

Example 1

Influence of genetic background on the efficiency of RNAi in *C. elegans*.

5 Introduction

Various different *C. elegans* strains were fed with different bacteria, to test the possibility of RNAi by feeding *C. elegans* with bacteria that produce dsRNA. The possibility of DNA delivery and dsRNA delivery has
10 previously been envisaged by using different bacterial strains. In this experiment the importance of the *C. elegans* strain as receptor of the dsRNA is also shown.

For this experiment the following *E. coli* strains were
15 used:

1. MC1061: F-*araD139* Δ (*ara-leu*)7696 *galE15 galK16*
 Δ (*lac*)X74 *rps1* (*Str^r*) *hsdR2* (*r_k⁻ m_k⁺*) *mcrA mcrB1*
- regular host for various plasmids,
20 - Wertman et al., (1986) Gene 49:253-262,
- Raleigh et al., (1989) in Current Protocols in Molecular Biology eds. Ausubel et al, Publishing associates and Wiley Interscience; New York. Unit 1.4.
- 25 2. B21(DE3): F- *ompT(lon)* *hsdS_B* (*r_B⁻, m_B⁻*; an *E. coli* B strain) with DE3, a λ prophage carrying the T7 RNA polymerase gene.
- regular host for IPTG inducible T7 polymerase
30 expression,
- Studier et al. (1990) Meth. Enzymol. 185:60-89
3. HT115 (DE3): F- *mcrA mcrb* IN(*rrnD-rrnE*) 1 λ -
rnc14::tr10 (DE3 lysogen: *lacUV5*
35 promoter-T7polymerase)
- host for IPTG inducible T7 polymerase

expression,
- RNaseIII-,
- Fire A, Carnegie Institution, Baltimore, MD,
Pers. Comm.

5

For this experiment the following *C. elegans* strains were used:

1. *C. elegans* N2: regular WT laboratory strain
2. *C. elegans* *nuc-1*(el393): *C. elegans* strain with a reduced endonuclease activity (>95%); condensed chromatin persists after programmed cell death; ingested (bacterial) DNA in the intestinal lumen is not degraded. Several alleles are described:
el392 (strong allele: has been used for the experiments described below); n887 (resembles el392) and n334 (weaker allele)
- Stanfield et al. (1998) East Coast Worm meeting abstract 171,
- Anonymous, Worm Breeder's Gazette 1(1):17b
Hevelone et al. (1988) Biochem. Genet. 26:447-461
- Ellis et al., Worm breeder's Gazette 7(2):44
- Babu, Worm Breeder's gazette 1(2):10
- Driscoll, (1996) Brain Pathol. 6:411-425
- Ellis et al., (1991) Genetics 129:79-94

For this experiment the following plasmids were used:

- pGN1: A vector encoding for ampicillin resistance, harbouring a multiple cloning site between two convergent T7 promoters.
- pGN8: pGN1 containing a genomic fragment of *unc-22*.
Decreased *unc-22* expression via RNAi results in a "twitching" phenotype in *C. elegans*.

Experimental conditions

12-well micro-titer plates were filled with approximately 2 ml of NGM agar per well (1 litre of NGM agar: 15g Agar, 1g peptone, 3g NaCl, 1ml cholesterol solution (5 mg/ml in EtOH), with sterile addition after autoclaving of 9.5 ml 0.1M CaCl₂, 9.5 ml 0.1 ml MgSO₄, 25 ml 1M KH₂PO₄/K₂HPO₄ buffer pH 6 and 5 ml nystatin solution (dissolved 10 mg/ml in 1:1 EtOH:CH₃COONH₄ 7.5 M).

The dried plates were spotted with approximately 50 µl of an overnight culture of bacteria. When IPTG induction was required, 50 µl of a 10 mM stock solution of IPTG was dropped on top of the bacteria lawn, and incubated at 37°C for approximately 4 hours. Individual nematodes at the L4 growth stage were then placed in single wells. In each well 4 nematodes, and the plates were further incubated at 20°C for 6 days to allow offspring to be formed. The F1 offspring of the seeded nematodes were tested for the twitching phenotype.

Results

Table 1: Percentage of the offspring that show the twitching phenotype

5	MC1061	N2	<i>nuc-1</i>
	pGN1	0%	0%
	pGN1 + IPTG	0%	0%
	pGN8	0%	0%
	pGN8 + IPTG	0%	0%
10	BL21 (DE3)		
	pGN1	0%	0%
	pGN1 + IPTG	0%	0%
	pGN8	20% (+)	>90% (++)
	pGN8 + IPTG	20% (+)	>90% (+++)
15	HT115 (DE3)		
	pGN1	0%	0%
	pGN1 + IPTG	0%	0%
	pGN8	50% (+±)	>90% (++)
20	pGN8 + IPTG	80% (++)	>90% (+++)

%: percentage twitchers

+: weak twitching

++: twitching

+++: strong twitching

Conclusions

The experiment with *E. coli* MC1061 shows that no twitching could be observed in this experiment.

Neither the N2 nematodes nor the *nuc-1* nematodes showed any twitchers. This is to be expected as *E. coli* MC1061 does not produce any T7 RNA polymerase, and hence the unc-22 fragment cloned in pGN8 is not

expressed as dsRNA.

5 The experiment with *E. coli* strain BL21(DE3) and
nematode strain N2 shows expected results. BL21(DE3)
harbouring plasmid pGN1 does not result in any
twitching as the pGN1 vector is an empty vector. BL21
(DE3) harbouring PGN8 results in the expression of
unc-22 dsRNA. When this dsRNA is fed to the N2
10 nematode (indirectly by feeding with the bacteria that
produce the dsRNA), this results in a twitching
phenotype, indicating that the dsRNA is able to pass
the gut barrier and is able to perform its interfering
activity.

15 Surprisingly the RNAi effect of the unc-22 dsRNA was
even more pronounced in *C. elegans* strain *nuc-1* than
in the wild type N2 strain. Although one may expect
that the *nuc-1* mutation results in the non-degradation
or at least in a slower degradation of DNA, as the
20 NUC-1 protein is known to be involved in DNase
activity, we clearly observe an enhancement of the
RNAi induced phenotype in *C. elegans* with a *nuc-1*
background. The *nuc-1* mutation has not been cloned
yet, but it has been described that the gene is
25 involved in nuclease activity, and more particularly
DNase activity. If the NUC-1 protein is a nuclease, it
may also have activity on nuclease activity on dsRNA,
which would explain the enhanced RNAi phenotype. The
nuc-1 gene product may be a nuclease, or a regulator
30 of nuclease activity. As the mode of action of RNAi is
still not understood, it is also possible that the
NUC-1 protein is interfering in the mode of action of
RNAi. This would explain why a *nuc-1* mutant is more
sensitive to RNAi.

35

The experiment with the *E. coli* strain HT115 (DE3)

confirms the experiments with the BL21(DE3) strain. The RNA interference observed with the unc-22 dsRNA is even higher. In comparison with strain BL21(DE3) this could be expected, as HT115(DE3) is a RNase III minus strain, and hence is expected to produce larger amounts of dsRNA, resulting in more prominent RNAi. This indicates further that the RNAi observed in this experiment is the result of the dsRNA produced by the bacteria fed to the *C. elegans*. Feeding *C. elegans* *nuc-1* with HT115(DE3) harbouring pGN8 also results in higher RNA interference phenotype than feeding the same bacteria to *C. elegans* wild-type strain N2. Once again this indicates that improved RNAi can realised using a nuclease negative *C. elegans* and more particularly with a with the *C. elegans nuc-1* (e1392) strain.

Summary

RNA interference can be achieved in *C. elegans* by feeding the worms with bacteria that produce dsRNA. The efficiency of this RNA interference is dependent both on the *E. coli* strain and on the genetic background of the *C. elegans* strain. The higher the level of dsRNA production in the *E. coli*, the more RNAi is observed. This can be realised by using efficient RNA expression systems such as T7 RNA polymerase and RNase negative strains, such as RNaseIII minus stains. In this example the level of dsRNA production varied: HT115(DE3)>BL21(DE3)>MC1061.

RNA interference is high in *C. elegans* strains that are nuclease negative, or that are influenced in their nuclease activity. This can be realised by using a mutant strain such as *C. elegans nuc-1*. In this example the sensitivity to RNAi varied: *C. elegans nuc-1* >> *C. elegans* N2

Example 2

Improved RNAi by feeding dsRNA producing bacteria in selected *C. elegans* strains-Comparison of the *nuc-1* strain with several mutants which show improved gut uptake (designated herein 'gun' mutants). Strains bg77, bg78, bg83, bg84, bg85, bg86, bg87, bg88 and bg89 are typical gun mutant *C. elegans* strains isolated using selection for increased gut uptake (gun phenotype) with the marker dye BCECF-AM.

Experimental conditions:

- 12-well micro-titer plates were filled with approximately 2ml of NGM agar (containing 1ml/l of ampicillin (100µg/ml) and 5 ml of 100mM stock IPTG) per well
- the dried plates were spotted with 25µl of an overnight culture of bacteria (BL21DE3/HT115DE3) containing the plasmids pGN1 (T7prom-T7prom) or pGN8 (T7prom-unc-22-T7prom)
- individual nematodes at the L4 growth stage were then placed in single wells, one nematode per well
- the plates were incubated at 20°C for 6 days to allow offspring to be formed
- the adult F1 offspring of the seeded nematodes were tested for the twitching phenotype

Results:

Table 2:

	20°C/6d	pGN1 HT115DE3	pGN8 BL2DE3	pGN8 HT115DE3	
5	N2	0	1	1	
	<i>nuc-1</i>	0	1-2	3	
	bg77	0	1-2	3	
	bg78	0	1	1-2	
	bg83	0	1	1	
10	bg84	0	1-2	3	
	bg85	0	1	2-3	
	bg86	0	1	2	
	bg87	0	1	1	
	bg88	0	1	1	
15	bg89	0	1	1	

figure legend:

- 0 = no twitching
- 1 = no to weak phenotype
- 2 = clear phenotype
- 3 = strong phenotype

25 **Conclusions**

- bacterial strain HT115(DE3) shows a better RNAi sensitivity than bacterial strain BL21(DE3)
- the *nuc-1* *C. elegans* strain is a better strain than the Wild-type N2 strain for RNAi sensitivity
- 30 - various gun mutants (improved gut uptake mutants) and more particularly the gun mutant strains bg77, bg84, bg85, bg86 show improved sensitivity to RNAi compared to Wild-type.

A double mutant *C. elegans* strain (nuc-1/gun) shows even greater sensitivity to RNAi compared to wild-type:

5 Double mutants were constructed to test the prediction
that gun/nuc mutants would even show more enhanced
RNAi sensitivity. As an example, the crossing
strategy with gun strain bg85 is shown, similar
crosses can be conducted with other gun strains, such
10 as bg77, bg84 and bg86.

P0 cross: gun(bg85) x WT males

15 F1 cross: nuc-1 x gun(bg85)/+ males

F2 cross: nuc-1 x gun(bg85)/+; nuc-1/0 males (50%)
nuc-1 x +/+; nuc-1/0 males (50%)

20 F3 single: gun(bg85)/+; nuc-1 hermaphrodites (25%)
+/+; nuc-1 hermaphrodites (75%)

F4 single: gun(bg85); nuc-1 (1/4 of every 4th
plate high staining with BCECF)

25 F5 retest: gun(bg85); nuc-1 (100% progeny of F4
singled high staining with BCECF)

To select for the gun phenotype, the fluorescence
precursor BCECF-AM is used (obtainable from Molecular
30 probes). The precursor BCECF-AM is cleaved by
esterases present in the gut of the worm to generate
the dye BCECF which is fluorescent at pH values above
6. This allows selection for worms that have a gun
phenotype. BCECF-AM is taken up through the pharynx
35 into the gut lumen and is not fluorescent until it has
been cleaved, and the BCECF portion has entered the

cells surrounding the lumen. Wild-type worms will show slower or no increase in BCECF fluorescence.

5 **Example 3**

Improved RNAi feeding in liquid culture using *nuc-1(e1393)* *C. elegans*.

Introduction

10 N2 and *nuc-1* *C.elegans* strains were fed with bacteria producing dsRNAs that give lethal phenotypes via RNAi. For this example RNAi was performed in liquid culture instead of on agar plates. We show here for a number of genes that the RNAi effect is more penetrant using
15 the *nuc-1* strain than the N2 strain, and that RNAi can be performed in liquid.

For this experiment the following *E.coli* strains were used:

20

1. HT115 (DE3): F- *mcrA mcrb IN(rrnD-rrnE)* 1 λ -
 rnc14::tr10 (DE3 lysogen: lacUV5 promoter -T7
 polymerase)
 - host for IPTG inducible T7 polymerase expression
25 - RNaseIII⁻
 - Fire A, Carnegie Institution, Baltimore, MD,
 Pers. Comm.

25

For this experiment, following *C. elegans* strains were
30 used:

30

1. *C. elegans* N2: regular WT laboratory strain
2. *C. elegans nuc-1(e1393)*: *C. elegans* strain with a
35 reduced endonuclease activity (>95%); condensed
 chromatin persists after programmed cell death;

35

- ingested (bacterial) DNA in the intestinal lumen is not degraded. Several alleles are described: e1392 (strong allele: has been used for the experiments described below); n887 (resembles e1392) and n334 (weaker allele)
- Stanfield et al. (1998) East Coast Worm meeting abstract 171
 - Anonymous, Worm Breeder's Gazette 1(1):17b
 - Hevelone et al. (1988) Biochem. Genet. 26:447-461
 - Ellis et al., Worm breeder's Gazette 7(2):44
 - Babu, Worm Breeder's gazette 1(2):10
 - Driscoll, (1996) Brain Pathol. 6:411-425
 - Ellis et al., (1991) Genetics 129:79-94

15

For this experiment, the following plasmids that all give lethal phenotypes in *C. elegans* via RNAi were used:

20 pGX22: a vector encoding ampicillin resistance, containing a genomic fragment of cosmid C04H5.6 corresponding to a member of the RNA helicase family.

25 pGX52: a vector encoding ampicillin resistance, containing a genomic fragment of cosmid K11D9.2b corresponding to sarco/endoplasmic Ca²⁺ ATPase also known as SERCA.

30 pGZ18: a vector encoding ampicillin resistance, containing a genomic fragment of cosmid T25G3.2 corresponding to a chitin like synthase gene.

35 pGX104: a vector encoding ampicillin resistance, containing a genomic fragment of cosmid Y57G11C.15 corresponding to sec-61, a transport protein.

Experimental conditions

- 1 ml overnight cultures of HT115 (DE3) bacteria containing the plasmids pGX22, pGX52, pGZ18 or pGX104 respectively were pelleted and resuspended in S-complete medium, containing 1ml/l of ampicillin (100 µg/ml) and 1ml/l of 1000mM IPTG.
- 10 µl of this bacterial solution was transferred to a 96-well microtiter plate already filled with 100 µl S-complete containing 1ml/l of ampicillin (100 µg/ml) and 1ml/l of 1000mM IPTG.
- 3 nematodes at the L1 growth stage of N2 and nuc-1 strain were then placed in single wells, 3 L1's per well. Per experimental set up, 16 wells were used (n=16).
- the plates were incubated at 25°C for 5 days to allow offspring to be formed.
- the plates were visually checked and the following phenotypes could be scored per individual well:
 - no effect:** L1's developed to adults and gave normal offspring.
 - no F1 offspring:** L1's developed to adults and gave no offspring.
 - acute lethal:** original L1 did not mature and died.

Results

- The results of this experiment are illustrated graphically in Figures 9 to 12. Data are expressed as

a percentage of the total (n=16) on the y-axis for both N2 and *nuc-1* strains.

Conclusions

5 The following genes were tested in this liquid RNAi assay:

- C04H5.6: an RNA helicase. RNAi of this gene interferes with the generation of offspring.
- 10 - SERCA: a sarco/endoplasmic Ca^{2+} ATPase. A strong RNAi phenotype causes an acute lethal phenotype. A less penetrant RNAi effect results in loss of offspring.
- 15 - T25G3.2: a chitin like synthase gene. RNAi of this gene causes dead eggs.
- sec-61: a transport protein. A strong RNAi phenotype causes an acute lethal phenotype. A less penetrant RNAi effect results in loss of offspring.
- 20 - RNAi can be performed under liquid conditions.

As in the previous examples this set of experiments shows that the *nuc-1 C. elegans* strain is more sensitive to RNAi than the wild-type N2 strain. This is most clear for less penetrant phenotypes such as SERCA and chitin synthase. For strong RNAi phenotypes like the helicase and Sec-61 the difference between the N2 wild-type strain and the *nuc-1* stain is less pronounced.

Example 4**Cloning of pGX22, pGX52, pGZ18 and pGX104 for RNAi**

A set of primers for each gene was designed on the basis of sequence data available in the publicly accessible *C. elegans* sequence database (Acedb).

The cosmid names relate to:

1. **C04H5.6**=member of RNA helicase
2. **K11D9.2b**=SERCA
3. **Y57G11C.15**=transport protein sec-61
4. **T25G3.2**=chitin synthase like

The following primer sequences were designed:

1. **C04H5.6F** 5'-TGCTCAGAGAGTTTCTCAACGAACC-3'
C04H5.6R 5'-CAATGTTAGTTGCTAGGACCACCTG-3'
2. **K11D9.2bF** 5'-CAGCCGATCTCCGTCTTGTG-3'
K11D9.2bR 5'-CCGAGGGCAAGACAACGAAG-3'
3. **Y57G11C.15F** 5'-ACCGTGGTACTCTTATGGAGCTCG-3'
Y57G11C.15R 5'-TGCAGTGGATTGGGTCTTCG-3'
4. **T25G3.2F**
5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTATGCCAAGTACATGTGCGATTGCG-3'
T25G3.2R
5'-GGGGACCACTTTGTACAAGAAAGCTGGGTTGGAGAAGCATTCCGAGAGTTTG-3'

PCR was performed on genomic DNA of N2 strain *C. elegans* to give PCR products of the following sizes:

- 1326bp for C04H5.6
- 1213bp for K11D9.2b

1024bp for Y57G11C.15

1115bp for T25G3.2

5 The PCR fragments of C04H5.6, K11D9.2b and Y57G11C.15
were linked to *Bst*XI adaptors (Invitrogen) and then
cloned into the pGN29 vector cut with *Bst*XI. pGN29
contains two T7 promoters and two T7 terminators
flanking a cloning site which is adapted for
10 facilitated cloning of PCR fragments, comprising a
stuffer DNA flanked by two *Bst*XI sites (see schematic
Figure 3). The resulting plasmids were designated
pGX22 (C04H5.6), pGX52 (K11D9.2b) and pGX104
(Y57G11C.15).

15 The PCR fragment of T25G3.2 was cloned into pGN39 via
recombination sites based on the GATEWAY™ cloning
system (Life Technologies, Inc). pGN39 contains two
T7 promoters and two T7 terminators flanking a cloning
site which facilitates "High Throughput" cloning based
20 on homologous recombination rather than restriction
enzyme digestion and ligation. As shown schematically
in Figure 4, the cloning site comprises *att*R1 and
*att*R2 recombination sites from bacteriophage lambda
flanking a gene which is lethal to *E. coli*, in this
25 case the *ccd*B gene. This cloning site is derived from
the Gateway™ cloning system commercially available
from Life Technologies, Inc. The Gateway™ cloning
system has been extensively described by Hartley et
al. in WO 96/40724 (PCT/US96/10082).

30

Example 5

Selecting *C. elegans* mutations for increased gut uptake (gun) using marker dye BCECF-AM and *unc-31* as background.

5

The screen was performed in *unc-31(e928)* mutant background, to ensure high amounts of dye in the gut lumen, since *unc-31* mutations show constitutive pharyngeal pumping. The dye (BCECF-AM: 2',7' bis (2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethylester), obtained from Molecular Probes, is cleaved by intracellular esterases. Fluorescence accumulates in the gut cells upon passage through the apical gut membrane.

15

Mutagenesis

Day 1: *unc-31* L4 staged worms were mutagenised with EMS (final concentration 50 mM) for 4 hours

Day 2: P0 was divided over several large agar plates

Day 6: F1's were collected and dropped on large plates. The number of eggs the F1's laid were checked every hour and de F1's were removed when 10-20 eggs per F1 were counted

Day 10: F2 adults were collected and screened with BCECF-AM. Mutations with increased staining of the gut cells after 15-30 minutes exposure to the dye were selected and singled on small agar plates.

30

About 50 initial positives gave progeny which was retested with BCECF-AM (2x) and leucine CMB (1x) 9 of the 50 strains were kept (2 strains : 3 times positive, 7 other strains : twice positive)

35

Table 3: Isolation of mutations for increased staining with BCECF-AM

Total P0	Total F1	Total F2	screened chromosomes	number of strains isolated
(counted)	(estimated)	(calculated)	(estimated)	(counted)
2251	55618	222472	100000	9

Outcrossing, backcrossing and double construction

1. backcrossing *unc-31; gun* --> *unc-31; gun*
 - *unc-31; gun* x WT males
 - singled 2x5 WT hermaphrodites F1s (*unc-31/+;gun/+*)
 - singled 50 WT hermaphrodites F2s (1/4 homozygous)
 - select strains segregating 1/4 *unc*
 - stain *unc* strains with BCECF-AM
 - from positive strains pick *unc* homozygous
 - retest 100 % *unc* strains with BCECF-AM
 - kept 1 strain (backcrossed)
2. *unc-31* background was crossed out-->+; *gun*
 - *unc-31; gun* x WT males
 - singled 2x5 WT hermaphrodites F1s (*unc-31/+;gun/+*)
 - singled 50 WT hermaphrodites F2s (1/4 homozygous)
 - select strains which did not segregate *unc* F3s anymore
 - stain non *unc* strains with BCECF-AM
 - 7 positive strains were retested with BCECF-AM and finally 1 was selected and kept (outcrossed)
3. +; *gun* (1x outcrossed) were 2 times backcrossed-->+; *gun* (3x backcrossed)
 - *gun* x WT males
 - WT hermaphrodites x F1 males (*gun/+*)
 - singled 10 WT hermaphrodites F2s (1/2 heterozygous)
 - singled 50 WT hermaphrodites F3s (1/8 homozygous)

- 31 -

- stain strains with BCECF-AM- retested positives with BCECF-AM and finally 1 was selected and kept

4. *gun* (3x backcrossed) were crossed with *nuc-1*(X)
 5 mutant--> *gun; nuc-1*
 - *gun* x WT males
 - *nuc-1* x *gun/+* males
 - *nuc-1* x *gun/+; nuc-1/0* or *+/+; nuc-1/0* males
 - singled 10 WT hermaphrodite progeny (*nuc-1*
 10 homozygous, $\frac{1}{2}$ heterozygous *gun*)
 - singled 40 WT hermaphrodite progeny (1/8 homozygous *gun*)
 - stain strains with BCECF-AM
 - retested positives with BCECF-AM and finally 1 was
 15 selected and kept

Table 6: Strains derived from *gun* mutations

	<i>gun</i>	<i>unc-31; gun</i>		<i>unc-31; gun</i>		+, <i>gun</i>		<i>gun; nuc-1</i>
		original isolate		backcrossed (1x)		outcrossed (1x)		from 3x b.c.
	allele number	isolation number	strain number	isolation number	strain number	isolation number	strain number	strain number
	bg77	31.4	UG 510	31.4.46.1	UG 556	31.4.34	UG 563	UG 674
	bg78	37.5	UG 511	37.5.46.4	UG 557	37.5.15	UG 564	UG 675
	bg83	10.2	UG 543	10.2.11	UG 600	10.2.21	UG 586	UG 676
	bg84	7.2	UG 544	7.2.10	UG 601	7.2.15	UG 589	UG 677
	bg85	11.5	UG 545	11.5.29.2	UG 602	2x b.c.	UG 717	UG 775
	bg86	42.1	UG 546	42.1.4.5	UG 603	42.1.18	UG 587	UG 678
	bg87	7.1	UG 547	7.1.8.3	UG 604	7.1.22	UG 585	UG 679
	bg88	5.3	UG 548	5.3.9	UG 605	5.3.18	UG 584	UG 680
	bg89	23.4	UG 549	23.4.13.5	UG 606	23.4.3	UG 588	UG 671

SEQUENCE LISTING:

SEQ ID NO: 1 complete sequence of pGN1

5 SEQ ID NO: 2 complete sequence of pGN8

SEQ ID NO: 3 complete sequence of pGN29

SEQ ID NO: 4 complete sequence of pGN39

10 SEQ ID NO: 5 complete sequence of pGX22

SEQ ID NO: 6 complete sequence of pGX52

15 SEQ ID NO: 7 complete sequence of pGX104

SEQ ID NO: 8 complete sequence of pGZ8

SEQ ID NO: 9 primer C04H5.6F

20 SEQ ID NO: 10 primer C04H5.6R

SEQ ID NO: 11 primer K11D9.2bF

25 SEQ ID NO: 12 primer K11D9.2bR

SEQ ID NO: 13 primer Y57G11C.15F

SEQ ID NO: 14 primer Y57G11C.15R

30 SEQ ID NO: 15 primer T25G3.2F

SEQ ID NO: 16 primer T25G3.2R

Claims:

1. A method of inhibiting expression of a target gene in a nematode worm comprising feeding to
5 said nematode worm a food organism which is capable of producing a double-stranded RNA structure having a nucleotide sequence substantially identical to a portion of said target gene following ingestion of the food organism by the nematode, wherein the nematode
10 has a non wild-type genetic background selected to provide increased sensitivity to RNA interference as compared to wild type.

2. A method as claimed in claim 1 wherein the
15 nematode is a microscopic nematode.

3. A method as claimed in claim 2 wherein the nematode is from the genus *Caenorhabditis*.

20 4. A method as claimed in claim 3 wherein the nematode is *C. elegans*.

5. A method as claimed in any one of claims 1 to 4 wherein the nematode has a mutant genetic
25 background.

6. A method as claimed in claim 5 wherein the nematode is a mutant strain which exhibits reduced activity of one or more nucleases compared to wild
30 type.

7. A method as claimed in claim 6 wherein the nematode is *C. elegans* strain *nuc-1*.

35 8. A method as claimed in claim 5 wherein the nematode is a mutant strain which exhibits increased

gut uptake compared to wild type.

9. A method as claimed in claim 8 wherein the nematode is mutant *C. elegans* strain bg85.

5

10. A method as claimed in claim 5 wherein the nematode is a mutant strain having at least one mutation which results in reduced nuclease activity compared to wild type and at least one mutation which results in increased gut uptake compared to wild type.

10

11. A method as claimed in claim 10 wherein the nematode is a mutant *C. elegans* strain having the *nuc-1* mutation and the bg85 mutation.

15

12. A method as claimed in any one of the preceding claims wherein the food organism has been engineered to express a double-stranded RNA.

20

13. A method as claimed in any one of the preceding claims wherein the food organism is a bacterium.

14. A method as claimed in claim 13 wherein the food organism is *E. coli*.

25

15. A method as claimed in any one of the preceding claims wherein the food organism has been genetically modified to express a double-stranded RNA having a nucleotide sequence substantially identical to a portion of said target gene.

30

16. A method as claimed in claim 15 wherein the food organism contains a DNA vector, the vector comprising a promoter or promoters orientated relative to a DNA sequence such that they are capable of

35

initiating transcription of said DNA sequence to RNA capable of forming a double-stranded structure upon binding of an appropriate RNA polymerase to said promoter or promoters.

5

17. A method as claimed in claim 25 wherein the vector comprises two promoters flanking the DNA sequence.

10

18. A method as claimed in claim 26 wherein the two promoters are identical.

15

19. A method as claimed in claim 25 wherein the vector comprises a single promoter and further comprises said DNA sequence in a sense and an antisense orientation relative to said promoter.

20

20. A method as claimed in any one of claims 16 to 20 wherein the nematode or the food organism is adapted to express an RNA polymerase capable of initiating transcription from said promoter or promoters.

25

21. A method as claimed in any one of claims 16 to 20 wherein the RNA polymerase is T7, T3 or SP6 polymerase.

30

22. A method as claimed in any one of claims 1 to 21 wherein the step of feeding said food organism to said nematode worm is carried out in liquid culture.

35

23. A method of inhibiting expression of a target gene in a nematode worm comprising feeding to said nematode worm a food organism capable of producing a double-stranded RNA structure having a nucleotide sequence substantially identical to a

portion of said target gene following ingestion of the food organism by the nematode, wherein the food organism carries a modification selected to provide increased expression or persistence of the doubled-stranded RNA compared to a food organism which does not carry the modification.

24. A method as claimed in claim 23 wherein the food organism is a bacterium.

25. A method as claimed in claim 24 wherein the bacterium is an *E. coli* strain.

26. A method as claimed in claim 25 wherein the *E. coli* strain is an RNase III minus strain or any other RNase negative strain.

27. A method as claimed in any one of claims 23 to 26 wherein the step of feeding said food organism to said nematode worm is carried out in liquid culture.

28. A method of inhibiting expression of a target gene in a nematode worm comprising introduction of a DNA capable of producing a double-stranded RNA structure having a nucleotide sequence substantially identical to a portion of said target gene in said nematode, wherein the nematode is one which exhibits increased gut uptake compared to wild type.

29. A method as claimed in claim 28 wherein the nematode is a microscopic nematode.

30. A method as claimed in claim 29 wherein the nematode is from the genus *Caenorhabditis*.

31. A method as claimed in claim 30 wherein the

nematode is *C. elegans*.

32. A method as claimed in any one of claims 28
to 31 wherein the nematode has a mutant genetic
5 background.

33. A method as claimed in claim 32 wherein the
nematode is mutant *C. elegans* strain bg85.

10 34. A method as claimed in any one of claims 28
to 33 wherein the DNA capable of producing a double-
stranded RNA structure is a vector comprising a
promoter or promoters orientated relative to a DNA
sequence such that they are capable of initiating
15 transcription of said DNA sequence to RNA capable of
forming a double-stranded structure upon binding of an
appropriate RNA polymerase to said promoter or
promoters.

20 35. A method as claimed in claim 34 wherein the
vector comprises two promoters flanking the DNA
sequence.

25 36. A method as claimed in claim 35 wherein the
two promoters are identical.

30 37. A method as claimed in claim 34 wherein the
vector comprises a single promoter and further
comprises said DNA sequence in a sense and an
antisense orientation relative to said promoter.

35 38. A method as claimed in any one of claims 34
to 37 wherein the nematode is adapted to express an
RNA polymerase capable of initiating transcription
from said promoter or promoters.

39. A method as claimed in any one of claims 34

to 38 wherein the RNA polymerase is T7, T3 or SP6 polymerase.

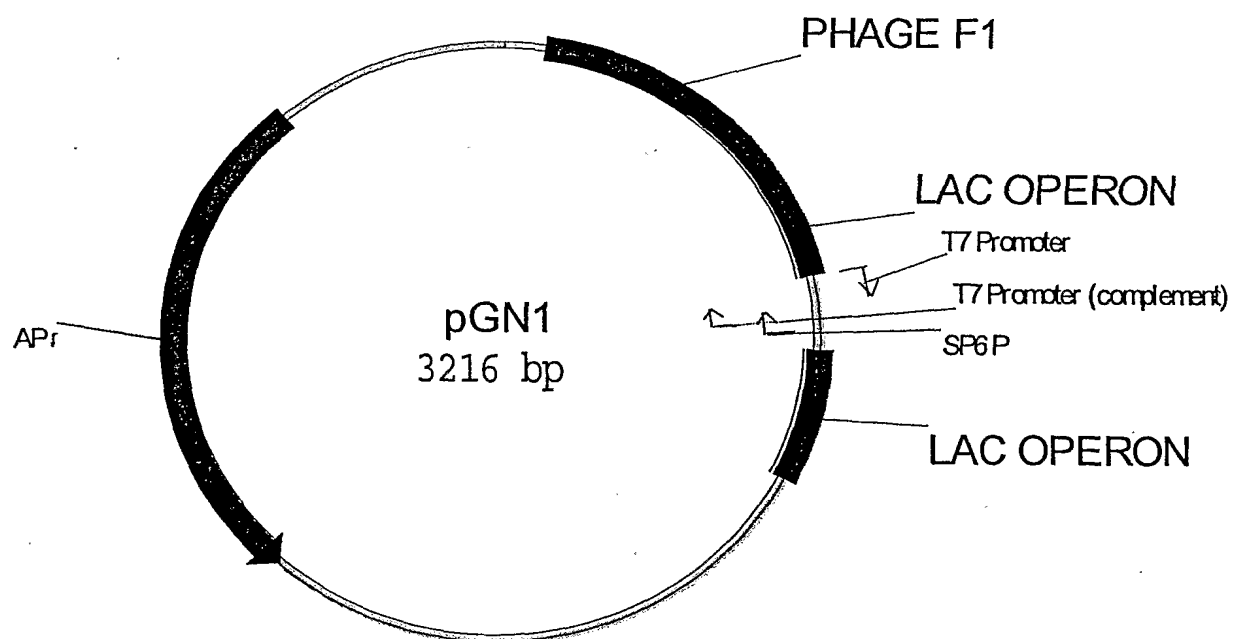
FIG. 1.

FIG. 2.

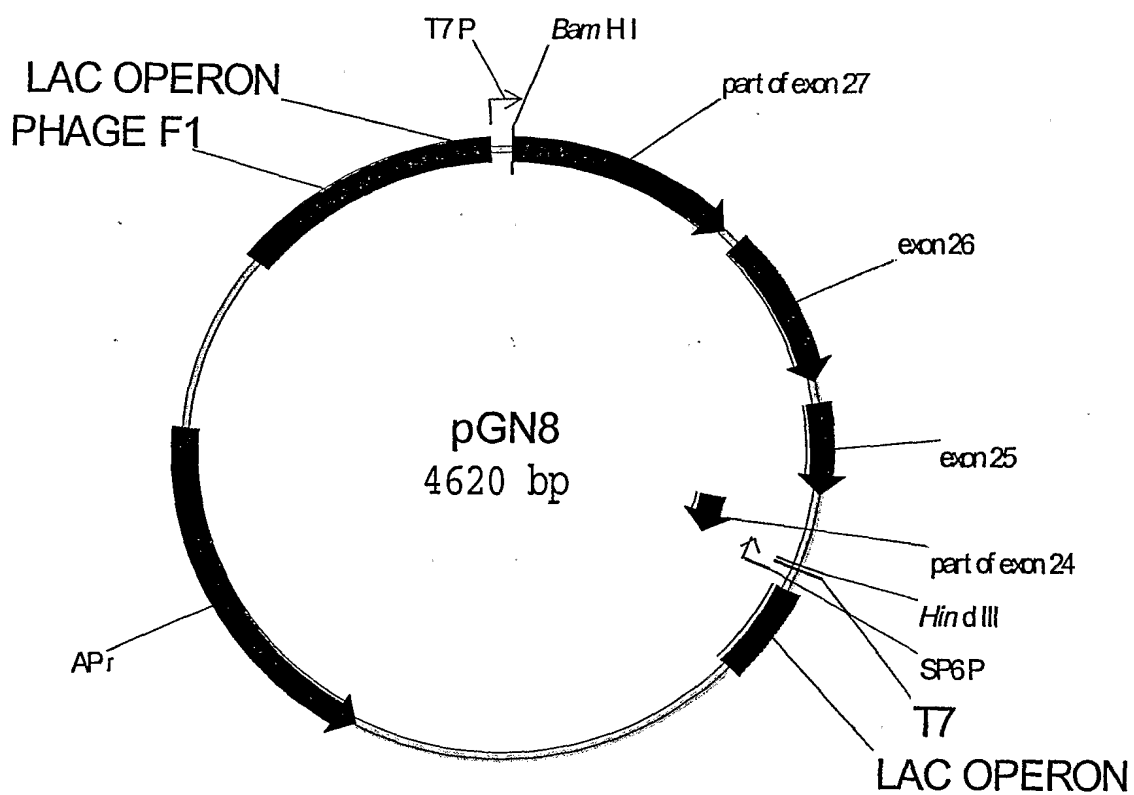


FIG. 3.

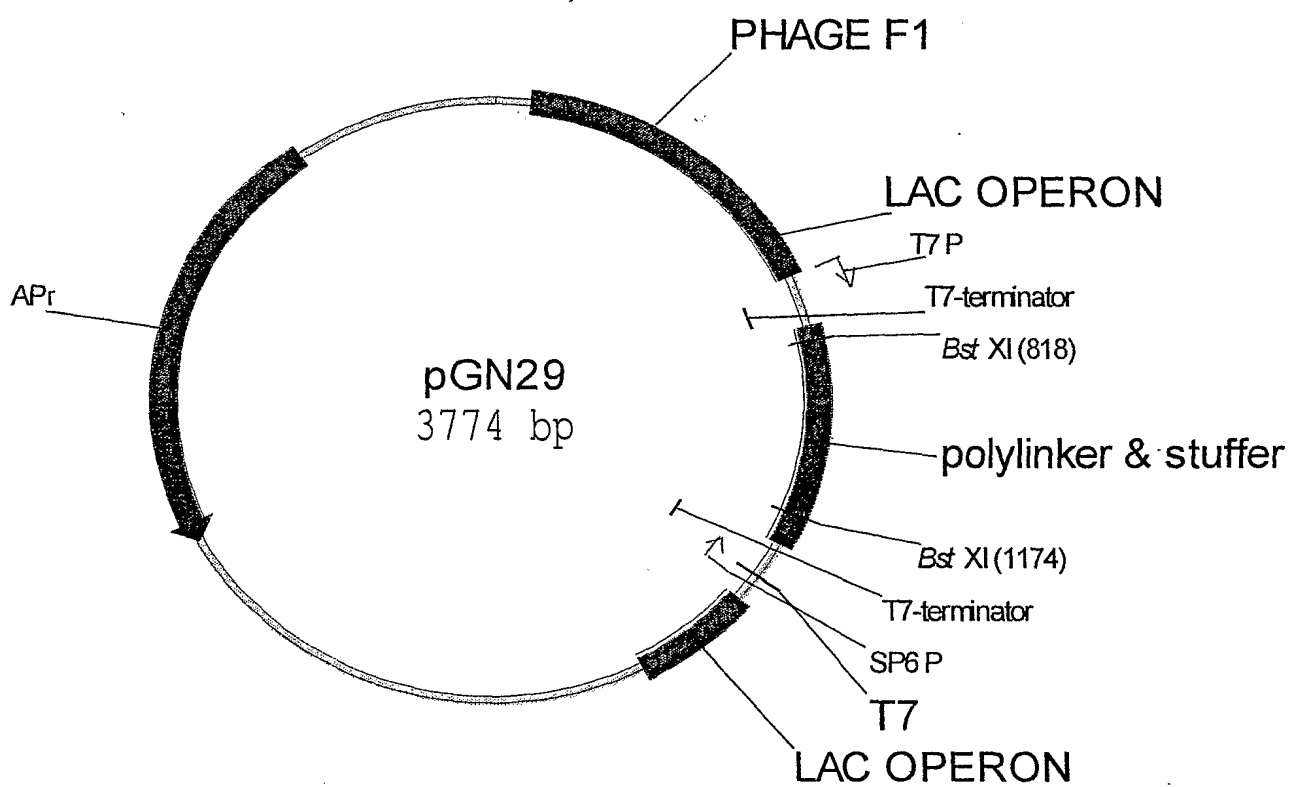


FIG. 4.

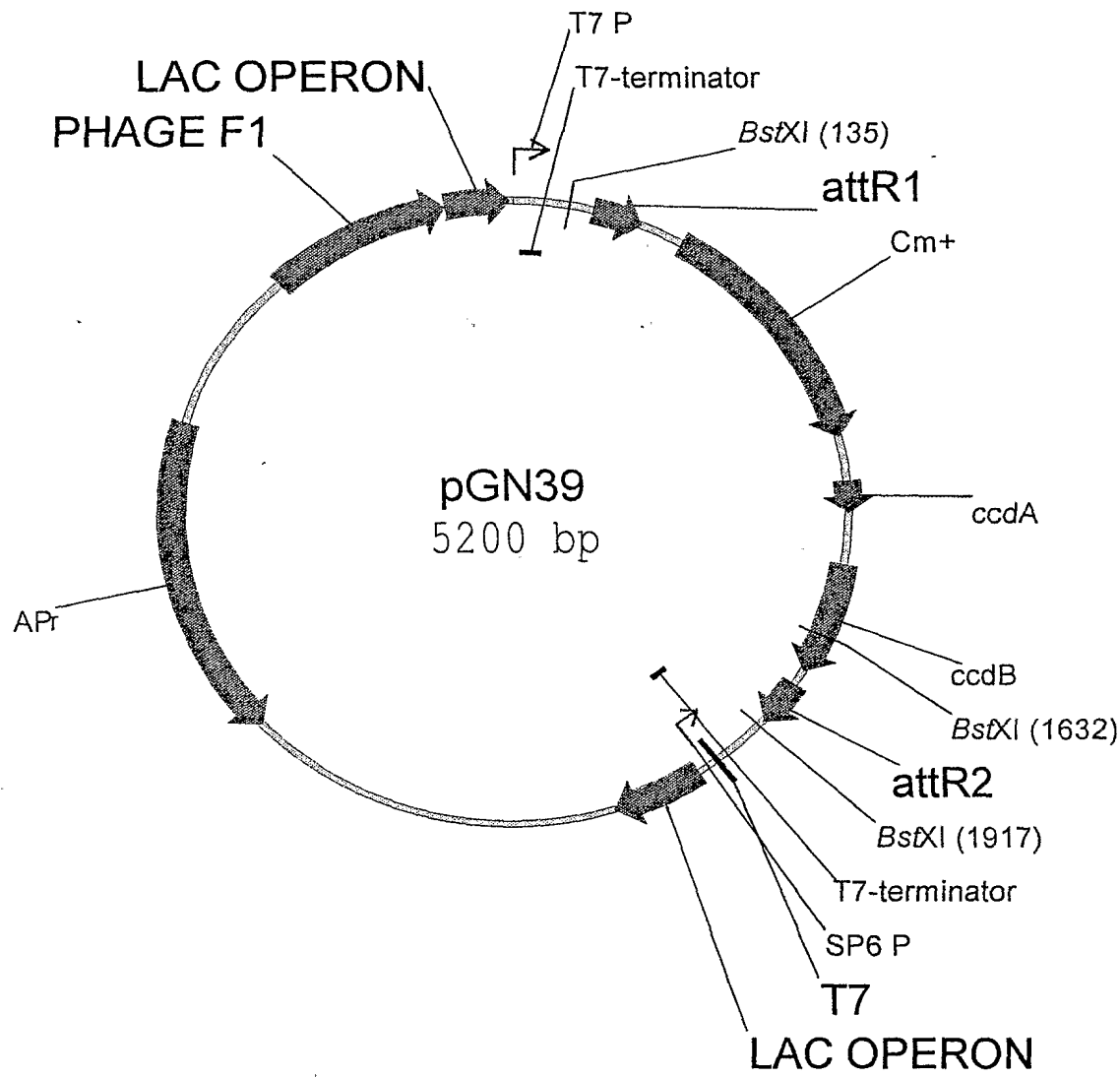


FIG. 5.

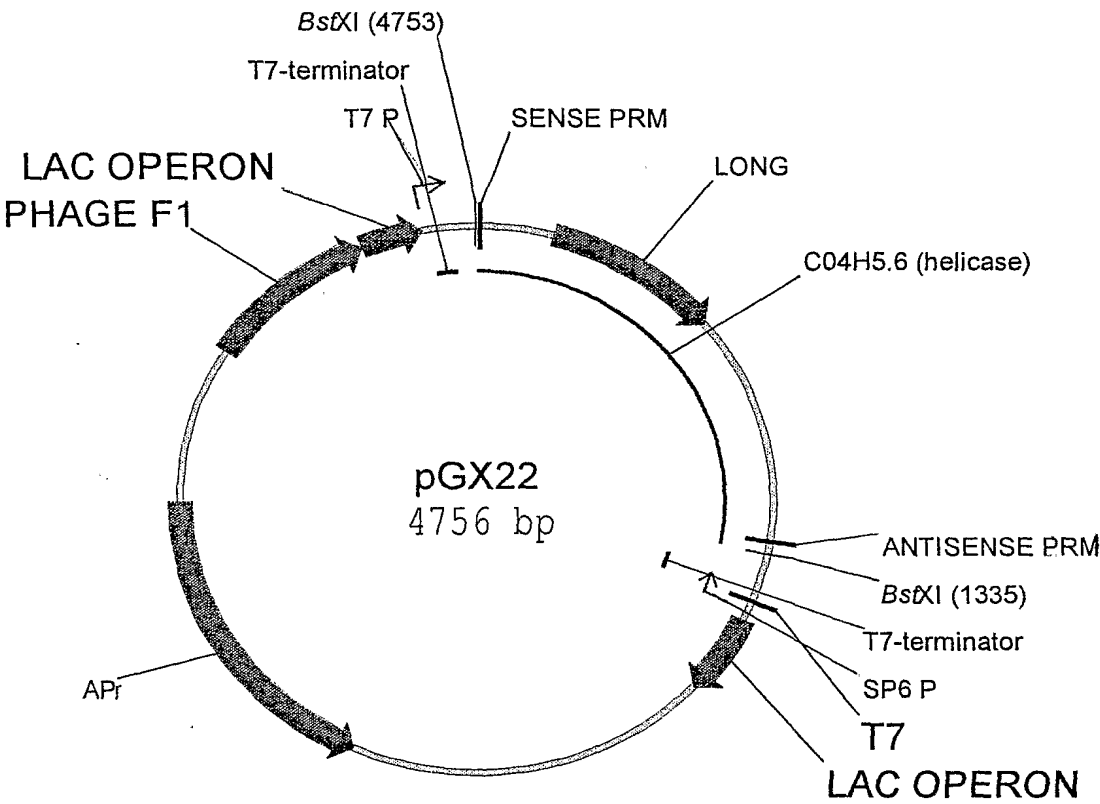


FIG. 6.

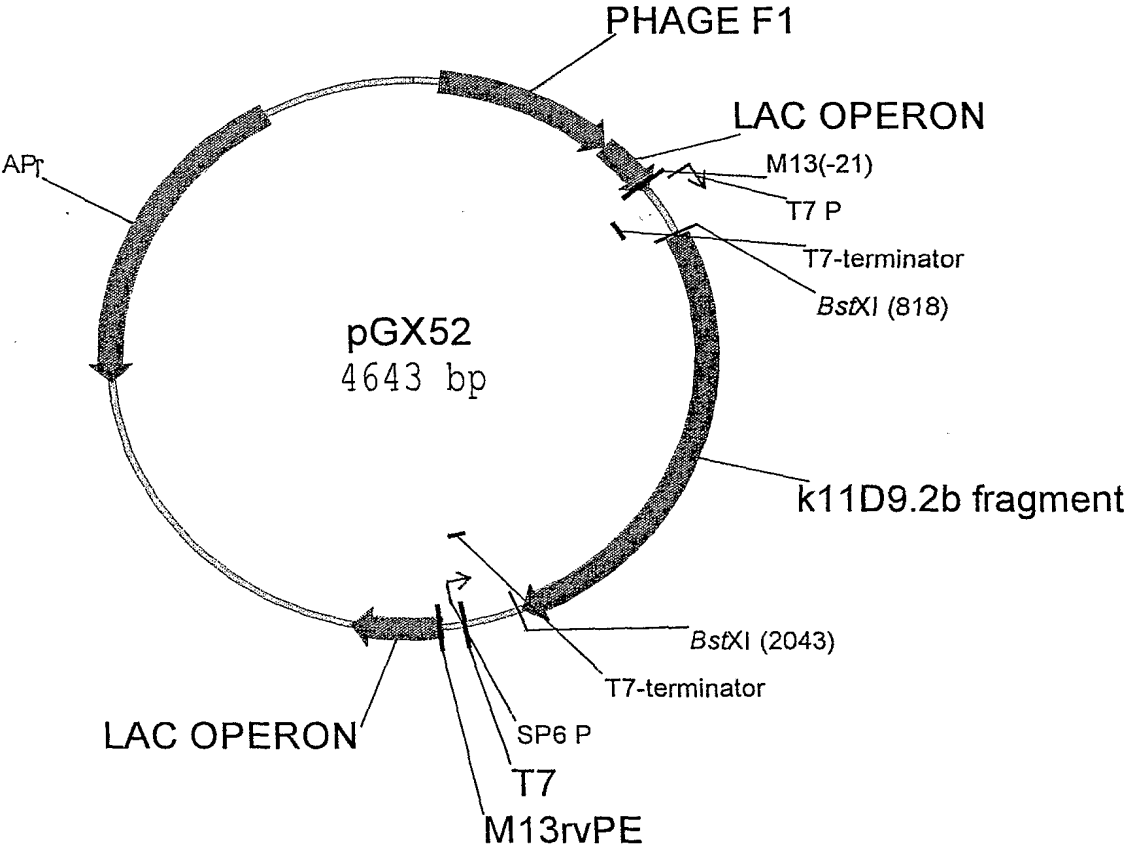


FIG. 7.

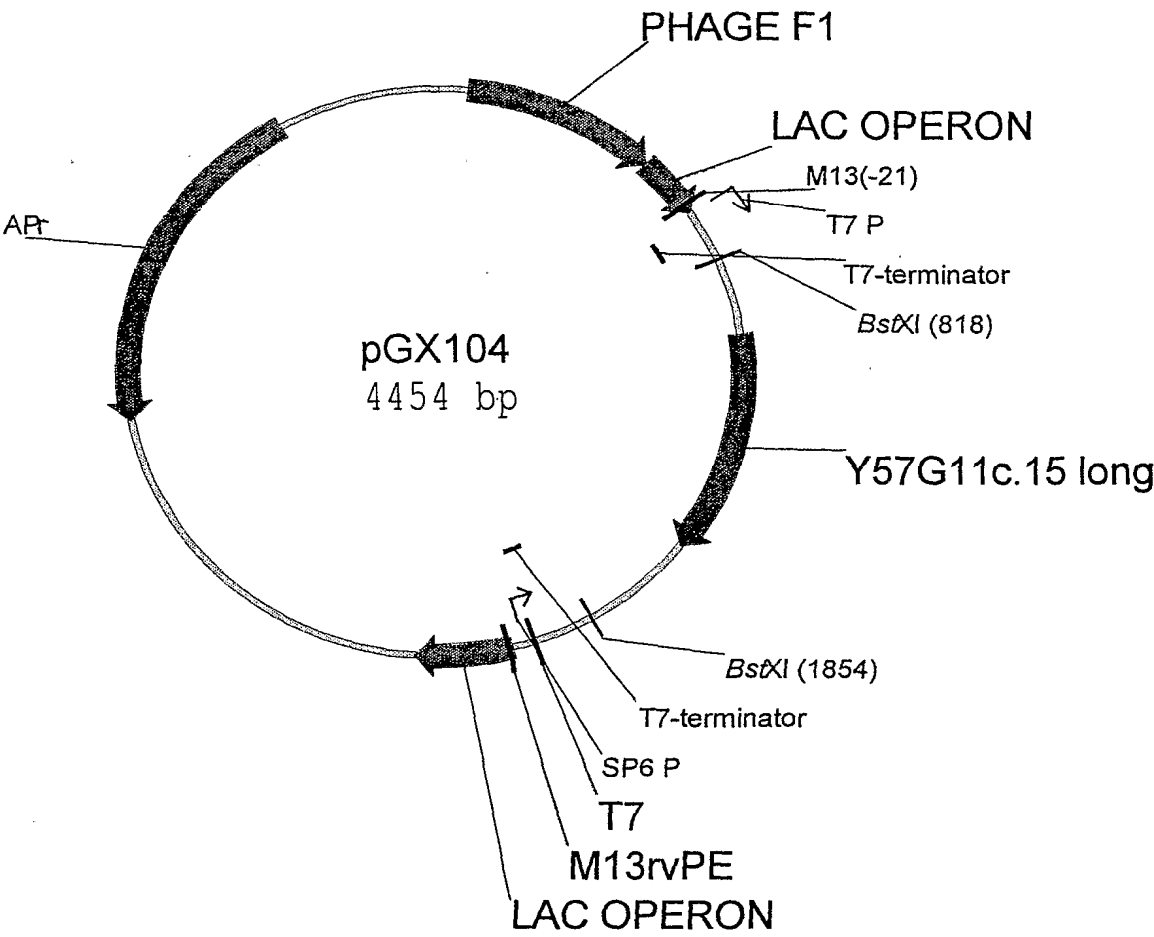


FIG. 8.

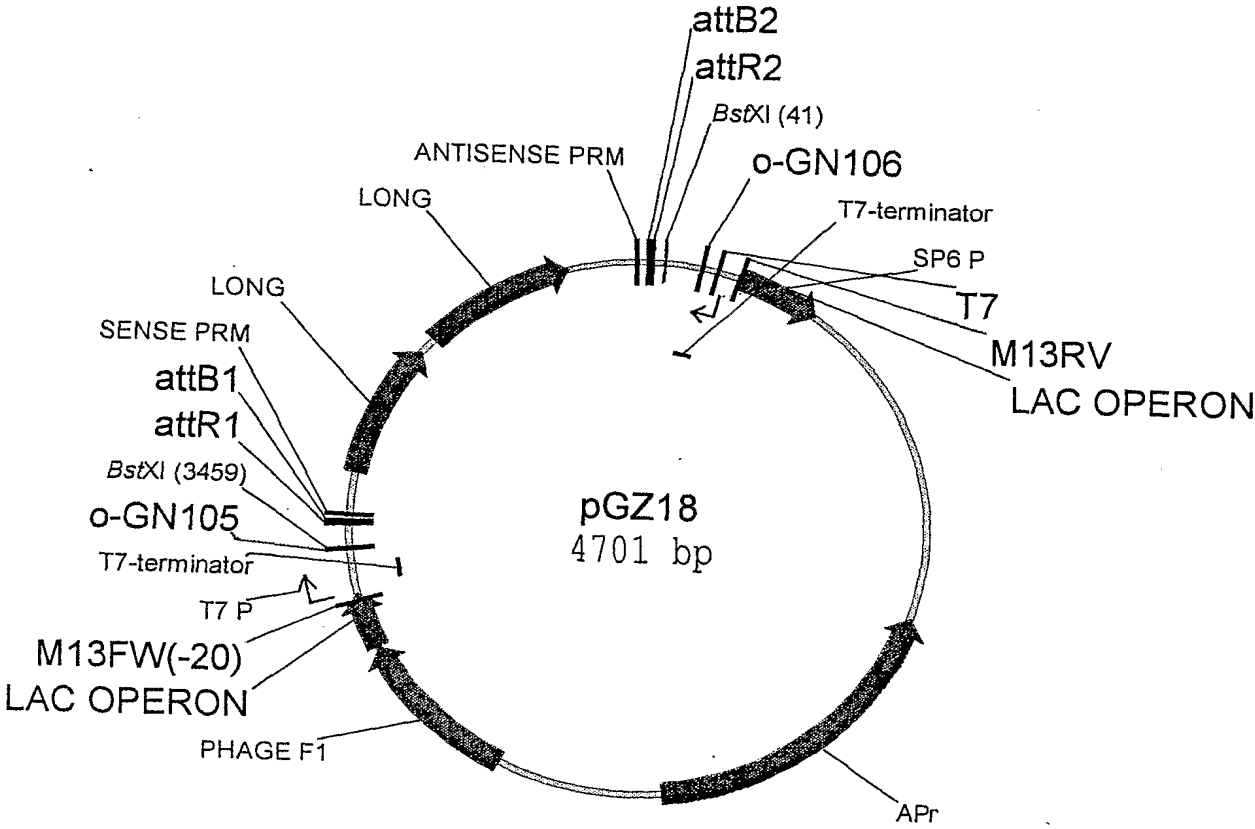


FIG. 9.

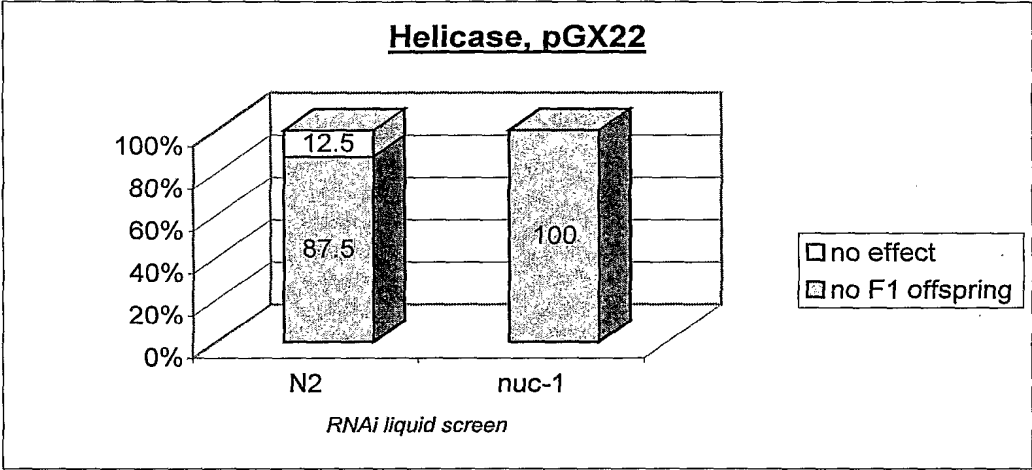


FIG. 10.

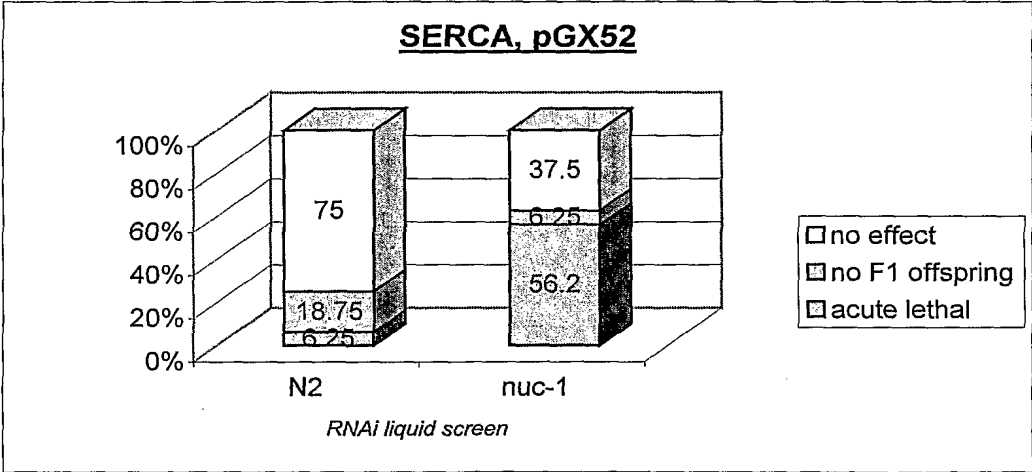


FIG. 11.

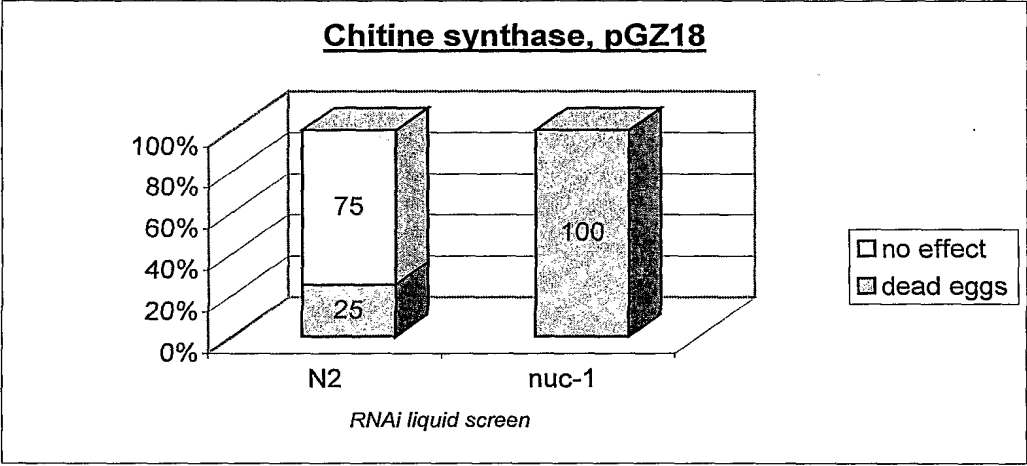
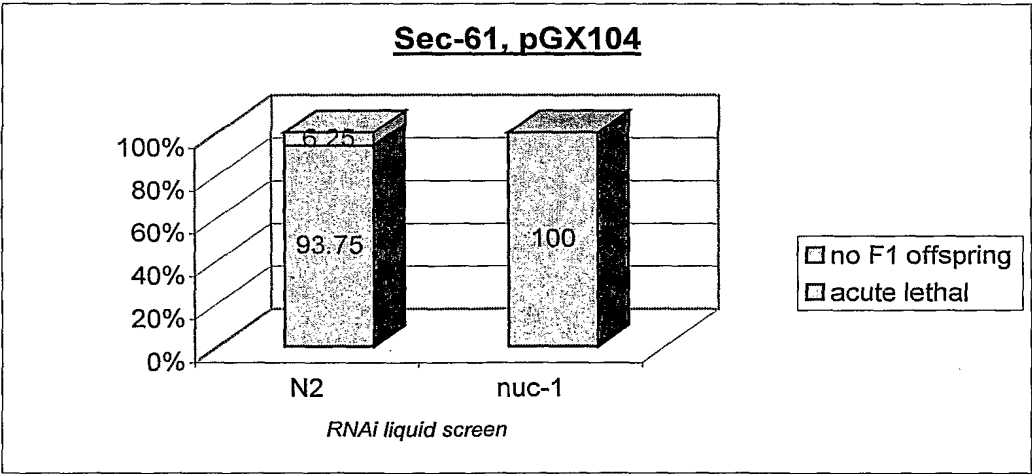


FIG. 12.



1
SEQUENCE LISTING

<110> DEVGEN NV

<120> IMPROVEMENTS RELATING TO DOUBLE-STRANDED RNA INHIBITION

<130> SCB/53711/001

<140>

<141>

<160> 14

<170> PatentIn Ver. 2.0

<210> 1

<211> 3216

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Plasmid pGN1

<400> 1

gagtgcacca	tatgcggtgt	gaaataccgc	acagatgcgt	aaggagaaaa	taccgcatca	60
ggcgaaattg	taaacgttaa	tattttgtta	aaattcgcgt	taaatatattg	ttaaatcagc	120
tcatttttta	accaataggc	cgaaatcggc	aaaatccctt	ataaatcaaa	agaatagacc	180
gagatagggt	tgagtgttgt	tccagtttgg	aacaagagtc	cactattaaa	gaacgtggac	240
tccaacgtca	aagggcgaaa	aaccgtctat	cagggcgatg	gcccactacg	tgaaccatca	300
cccaaataca	gttttttgcg	gtcgaggtgc	cgtaaagctc	taaatcggaa	ccctaaaggg	360
agcccccgat	ttagagcttg	acggggaaaag	ccggcgaaacg	tggcgagaaa	ggaagggaag	420
aaagcgaaag	gagcggggcgc	tagggcgctg	gcaagtgtag	cggtcacgct	gcgcgtaacc	480
accacacccg	ccgcgcttaa	tgcgcgcgta	cagggcgcgct	ccattcgcca	ttcaggctgc	540
gcaactgttg	ggaagggcga	tcggtgcggg	cctcttcgct	attacgccag	ctggcgaaaag	600
ggggatgtgc	tgcaaggcga	ttaagttggg	taacgccagg	gttttcccag	tcacgacggt	660
gtaaaacgac	ggccagtgaa	ttgtaatacg	actcactata	gggcgaattc	gagctcggta	720
ccgggggatc	ctctagagtc	gaaagcttct	cgccctatag	tgagtcgtat	tacagcttga	780
gtattctata	gtgtcaccta	aatagcttgg	cgtaatcatg	gtcatagctg	tttctctgtg	840
gaaattgtta	tccgctcaca	attccacaca	acatacgagc	cgggaagcata	aagtgtaaaag	900
cctgggggtgc	ctaattgagt	agctaactca	cattaattgc	gttgcgctca	ctgcccgcctt	960
tccagtcggg	aaacctgtcg	tgccagctgc	attaatgaat	cggccaacgc	gcggggagag	1020
gcggttttgc	tattgggcgc	tcttcgcgtt	cctcgctcac	tgactcgctg	cgctcggtcg	1080
ttcgggtgcg	gcgagcggta	tcagctcact	caaaggcgg	aatacggta	tccacagaat	1140
caggggataa	cgcaggaaaag	aacatgtgag	caaaaggcca	gcaaaaggcc	aggaaccgta	1200
aaaaggccgc	gttgctggcg	tttttcgata	ggctccgccc	ccctgacgag	catcacaaaa	1260
atcgacgctc	aagtcagagg	tggcgaaacc	cgacaggact	ataaagatac	caggcgtttc	1320
cccttggaag	ctccctcgtg	cgctctcctg	ttccgaccct	gccgcttacc	ggatacctgt	1380
ccgcctttct	cccttcggga	agcgtggcgc	tttctcatag	ctcacgctgt	aggtatctca	1440
gttcgggtgta	ggtcggtcgc	tccaagctgg	gctgtgtgca	cgaaccccc	gttcagcccc	1500
accgctgcgc	cttatccggt	aactatcgtc	ttgagtccaa	cccggtaaga	cacgacttat	1560
cgccactggc	agcagccact	ggtaacagga	ttagcagagc	gaggatgta	ggcggtgcta	1620
cagagttctt	gaagtgggtg	cctaactacg	gctacactag	aaggacagta	tttggtatct	1680
gcgctctgct	gaagccagtt	accttcggaa	aaagagttgg	tagctcttga	tccggcaaac	1740
aaaccaccgc	tggtagcgg	ggtttttttt	tttgcaagca	gcagattacg	cgcagaaaaa	1800
aaggatctca	agaagatcct	ttgatctttt	ctacggggtc	tgacgctcag	tggaacgaaa	1860
actcacgtta	agggatttt	gtcatgagat	tatcaaaaag	gatcttcacc	tagatccttt	1920
taaattaaaa	atgaagtttt	aaatcaatct	aaagtatata	tgagtaaact	tggtctgaca	1980

gttaccaatg	cttaatcagt	gaggcaccta	tctcagcgat	ctgtctatatt	cgttcatcca	2040
tagttgcctg	actccccgtc	gtgtagataa	ctacgatacg	ggagggctta	ccatctggcc	2100
ccagtgtgc	aatgataccg	cgagaccac	gctcacggc	tccagattta	tcagcaataa	2160
accagccagc	cggaagggcc	gagcgcagaa	gtggctctgc	aactttatcc	gcctccatcc	2220
agtctattaa	ttgttgccgg	gaagctagag	taagtagttc	gccagttaat	agtttgcgca	2280
acgttgttgg	cattgctaca	ggcatcgtgg	tgtcacgctc	gtcgtttggt	atggcttcat	2340
tcagctccgg	ttcccaacga	tcaaggcgag	ttacatgac	ccccatgttg	tgcaaaaaag	2400
cggttagctc	cttcgggtcct	cggatcgttg	tcagaagtaa	gttggccgca	gtgttatcac	2460
tcattggttat	ggcagcactg	cataattctc	ttactgtcat	gccatccgta	agatgctttt	2520
ctgtgactgg	tgagtactca	accaagtcac	tctgagaata	ccgcgcccgg	cgaccgagtt	2580
gctcttgccc	ggcgtcaata	cgggataata	gtgtatgaca	tagcagaact	ttaaaagtgc	2640
tcattcattgg	aaaacgttct	tcggggcgaa	aactctcaag	gatcttaccg	ctggtgagat	2700
ccagttcgat	gtaaccact	cgtgcaccca	actgatcttc	agcatctttt	actttcacca	2760
gcgtttctgg	gtgagcaaaa	acaggaaggc	aaaatgcgc	aaaaaaggga	ataagggcga	2820
cacggaaatg	ttgaatactc	atactcttcc	tttttcaata	ttattgaagc	atttatcagg	2880
gttattgtct	catgagcgga	tacatatttg	aatgtattta	gaaaaataaa	caaatagggg	2940
ttccgcgcac	atttccccga	aaagtgccac	ctgacgtcta	agaaaccatt	attatcatga	3000
cattaaccta	taaaaatagg	cgtatcacga	ggccctttcg	tctcgcgctg	ttcgggtgatg	3060
acggtgaaaa	cctctgacac	atgcagctcc	cggagacggg	cacagcttgt	ctgtaagcgg	3120
atgccgggag	cagacaagcc	cgtcagggcg	cgtcagcggg	tgttggcggg	tgtcggggct	3180
ggcttaacta	tgcgccatca	gagcagattg	tactga			3216

<210> 2

<211> 4620

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Plasmid pGN8

<400> 2

gatccgaatc	tccatgtctg	ttaacagcct	tgacacggaa	tttatattca	tgcccttgag	60
tcaaatecgtc	aacgtggaag	ttggtatcct	tgctctctcc	gcaagcagtc	catctgccag	120
tggcagcatc	ttgcttttca	atgacatagt	gactgatttc	agctcctcca	tcattctctg	180
gttccttcca	tgcaagatca	catccatcct	tgacaatatt	agtgacatcg	agaggtccac	240
gtgggcttga	tggatgatca	agaacagtaa	ccttcacttc	agcagtgctca	gttccattct	300
cgttctctgc	cttgatgata	taggttctctg	tatccgaacg	caaagctctc	ttcacatgga	360
atthagtctt	gccgtcttca	ttgttcaact	tcatacgatc	atcagattcg	actgggtgttc	420
cttcgaaagt	ccaagtaatt	gttggagttg	gttcaccact	gactggaatg	ttcaatgaga	480
agctcttgcc	agccttgacc	ttgatttctt	gaattcgagt	acgatcgatg	actgggtggaa	540
ctataatttta	attcaatgat	tattagtaatt	tgatttagac	tcttaccatt	tctagccttt	600
gcaacagctg	atgctgaatc	agatggatct	cccaatcctg	ccttggttctt	ggcacggatt	660
ctgaattcgt	actttgatcc	ttccttgaga	tttccaacag	tagcattcgt	ttgtccagct	720
ggaacatgag	caacgtcatt	ccagaatggc	gagaactcgt	ccttcacttc	aacaacgtat	780
tcctcgattg	gagcaccacc	gtcgtttgct	ggtggcttcc	attcaaggtc	aacatgatcc	840
ttatcccaat	cagtaatttc	aggagcattt	gtctttcctg	gcttgtcaaa	tggtatctttg	900
gcaagtgtgg	ttccgaaggt	ctccaatgga	tcggactctc	cttcagcatt	gacggcagcg	960
acacggaact	gaaaatcaaa	atgttgtagg	caattgagtt	caagattaaa	aaattctcac	1020
tttatattca	tgtccaggaa	taagaccgtc	aacaacagct	gtagtcttat	ctccagcgac	1080
ctttgcagct	ggaacccatc	ttccacttgc	agtatcgtac	ttttcgatca	catagttttc	1140
aattggaata	cctccatcat	catctgggtg	acgccaatc	aaagtgacat	gatcaccatg	1200
aacatcgga	acatctaattg	gaccatttgg	agaagttggc	ttgtctgaaa	atttaaaata	1260
taaccaaat	aattgaagaa	aactaatgct	caccaataac	attgatctta	acagttgctt	1320
catcttctcc	atlttgcatgg	acagctttga	tagtgaaagt	tccactgtct	ccagttcca	1380
tttgctttcac	aaccagcttt	gattgggtatt	ctgggttatc	aagcttctcg	ccctatagtg	1440
agtcgtatta	cagcttgagt	attctatagt	gtcacctaaa	tagcttggcg	taatcatggt	1500
catagctgtt	tcctgtgtga	aattgttatc	cgtccacaat	tccacacaac	atacgagccg	1560
gaagcataaa	gtgtaaagcc	tgggggtgcct	aatgagtgag	ctaactcaca	ttaattgcgt	1620

tgcgctcact	gcccgcctttc	cagtcgggaa	acctgtcgtg	ccagctgcat	taatgaatcg	1680
gccaacgcgc	ggggagaggc	ggtttgcgta	ttgggcgctc	ttccgcttcc	tcgctcactg	1740
actcgctgcg	ctcggtcgtt	cggctgcggc	gagcggatc	agctcactca	aaggcggtaa	1800
tacggttatc	cacagaatca	ggggataacg	caggaaagaa	catgtgagca	aaaggccagc	1860
aaaaggccag	gaaccgtaaa	aaggccgcgt	tgctggcggt	tttcgatagg	ctccgcccc	1920
ctgacgagca	tcacaaaaat	cgacgctcaa	gtcagagggtg	gcgaaacccg	acaggactat	1980
aaagatacca	ggcgtttccc	cctggaagct	ccctcgtgcg	ctctcctggt	ccgaccctgc	2040
cgcttaccgg	atacctgtcc	gcctttctcc	cttcgggaag	cgtggcgctt	tctcatagct	2100
cacgctgtag	gtatctcagt	tcgggtgtagg	tcgttcgctc	caagctgggc	tgtgtgcacg	2160
aacccccgt	tcagcccagc	cgctgcgcct	tatccggtaa	ctatcgtctt	gagtccaacc	2220
cggtaagaca	cgacttatcg	ccactggcag	cagccactgg	taacaggatt	agcagagcga	2280
ggatgttagg	cgggtgctaca	gagttcttga	agtgggtggc	taactacggc	tacactagaa	2340
ggacagtatt	tggtatctgc	gctctgctga	agccagttac	cttcggaaaa	agagttggta	2400
gctcttgatc	cggcaaacaa	accaccgctg	gtagcgggtg	tttttttgtt	tgcaagcagc	2460
agattacgcg	cagaaaaaaa	ggatctcaag	aagatccttt	gatcttttct	acggggtctg	2520
acgctcagtg	gaacgaaaac	tcacgttaag	ggatttttgt	catgagatta	tcaaaaagga	2580
tcttcaccta	gatcctttta	aattaaaaat	gaagttttta	atcaatctaa	agtatatatg	2640
agtaaacttg	gtctgacagt	taccaatgct	taatcagtga	ggcacctatc	tcagcgatct	2700
gtctatttcg	ttcatccata	gttgccctgac	tccccgtcgt	gtagataact	acgatacggg	2760
agggttacc	atctggcccc	agtgtctgca	tgataaccgcg	agaccacgc	tcaccggctc	2820
cagatttatc	agcaataaac	cagccagccg	gaagggccga	gcgcagaagt	ggtcctgcaa	2880
ctttatccgc	ctccatccag	tctattaatt	gttgccggga	agctagagta	agtagttcgc	2940
cagttaatat	tttgcgcaac	gttggttgca	ttgctacagg	catcgtgggtg	tcacgctcgt	3000
cgtttggtat	ggcttcattc	agctccgggt	cccaacgatc	aaggcgagtt	acatgatccc	3060
ccatgttggtg	caaaaaagcg	gttagctcct	tcgggtcctcc	gatcgttggtc	agaagtaagt	3120
tgccgcagtg	gttatcactc	atggttatgg	cagcactgca	taattctctt	actgtcatgc	3180
catccgtaag	atgcttttct	gtgactgttg	agtactcaac	caagtcattc	tgagaatacc	3240
gcgcccggcg	accaggttgc	tcttgcccgg	cgtcaatacg	ggataatagt	gtatgacata	3300
gcagaacttt	aaaagtgttc	atcattggaa	aacgttcttc	ggggcgaaaa	ctctcaagga	3360
tcttaccgct	gttgagatcc	agttcgatgt	aaccactcgc	tgcaaccaac	tgatcttcag	3420
catcttttac	tttcaccagc	gtttctgggt	gagcaaaaac	aggaaggcaa	aatgccgcaa	3480
aaaagggaat	aagggcgaca	cggaaatgtt	gaatactcat	actcttcctt	tttcaatatt	3540
attgaagcat	ttatcagggt	tattgtctca	tgagcggata	catatttgaa	tgtatttaga	3600
aaaataaaca	aataggggtt	ccgcgcacat	ttccccgaaa	agtgccacct	gacgtctaag	3660
aaaccattat	tatcatgaca	ttaacctata	aaaataggcg	tatcacgagg	ccctttcgtc	3720
tcgcgcggtt	cgggtgatgac	ggtgaaaacc	tctgacacat	gcagctcccg	gagacgggtca	3780
cagcttgtct	gtaagcggat	gccgggagca	gacaagcccg	tcagggcgcg	tcagcgggtg	3840
ttggcgggtg	tcggggctgg	cttaactatg	cggcatcaga	gcagattgta	ctgagagtgc	3900
accatatgcg	gtgtgaaata	ccgcacagat	gcgtaaggag	aaaataccgc	atcaggcgaa	3960
attgtaaacg	ttaatathtt	gttaaaattc	gcgttaaata	tttgttaaat	cagctcattt	4020
tttaaccaat	aggccgaat	cggcaaaatc	ccttataaat	caaaagaata	gaccgagata	4080
gggttgagtg	ttgttccagt	ttggaacaag	agtcactat	taaagaacgt	ggactccaac	4140
gtcaaagggc	gaaaaaccgt	ctatcagggc	gatggcccac	tacgtgaacc	atcacccaaa	4200
tcaagttttt	tgcggtcagag	gtgccgtaaa	gctctaaatc	ggaaccctaa	agggagcccc	4260
cgatttagag	cttgacgggg	aaagccggcg	aacgtggcga	gaaaggaagg	gaagaaagcg	4320
aaaggagcgg	gcgctagggc	gctggcaagt	gtagcgggtca	cgctgcgcgt	aaccaccaca	4380
cccgcgcgc	ttaatgcgcc	gctacagggc	gcgtccattc	gccattcagg	ctgcgcaact	4440
gttgggaagg	gcgatcgggtg	cgggcctctt	cgctattacg	ccagctggcg	aaagggggat	4500
gtgctgcaag	gcgattaagt	tgggtaacgc	caggggtttc	ccagtcacga	cgttgtaaaa	4560
cgacggccag	tgaattgtaa	tacgactcac	tatagggcga	attcgagctc	ggtacccggg	4620

<210> 3

<211> 4756

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Plasmid pGX22

<400> 3

tgctcagaga	gtttctcaac	gaacccgatt	tggctagtta	taggtaat	ttagaacatt	60
tacaaaaaca	gcaaaaaaac	caaacattca	ggatttttgt	ttttaattaa	gaaaaaaatc	120
gatcgctctt	aaattttaat	caatacttcg	aataaaccca	aaaaaaaacg	aaaaaaaatc	180
ctgtttccag	tgtaatgatg	attgacgagg	ctcacgaacg	tactctacac	acggatattc	240
tattcgggtt	agtcaaagat	attgcaagat	tccgaaagga	tttgaagctt	ctcatctctt	300
ctgcaacact	tgacgctgaa	aagttctcca	gtttcttcga	cgacgctccg	attttccgaa	360
ttccggggacg	cagattccccg	gtggacattt	actatacaca	ggctcccga	gcggaactacg	420
tcgacgcggc	tatcgtcaca	attatgcaga	ttcacttgac	ccagccactt	cccggcgata	480
ttttgggtatt	tctgactggg	caggaagaaa	tcgaaactgt	acaggaagca	cttatggaac	540
ggtcgaaagc	actgggatcg	aagattaagg	agctttattcc	gctgccgggt	tatgcgaatt	600
tgcccagtg	tttgaggcg	aagatttttcg	agccaacgcc	gaaagatgcg	agaaaggtag	660
atttttctta	caaatttttt	caaaaaaaa	atccgagaaa	aatctacaaa	atttcaggca	720
aaaactgttt	caattttattc	ctaactagtt	ttttagcaaa	cgttttagatt	taacaaaaact	780
gaacaaat	gaagttttcc	aatttataaaa	ataaatgttt	cggaaagt	attgaaaaat	840
ctgaaattgc	tatcctctcg	tatctgcaaa	aaaaacactt	taaaaaatgc	tctgttcttt	900
gaaattttct	aaactgaaaa	atttgaaatt	tctgaaaatt	gtgataat	tataaaat	960
tatagaaaat	gtaagcattc	cagaaaaata	tcaaaaat	cgagaaaatt	ctgaaaaaat	1020
ccagaaatat	taacagaaaa	aaaatctttt	gaaacatctg	aaaattaaaa	taaattgaat	1080
ttacattttt	ttttttggga	tttccttaaa	atcactatga	atttaccact	aaattttttg	1140
caaaaaatta	tttttttaat	ttcaaagaaa	aagcaaagaa	ttttaaaata	tcaaaaagtc	1200
caaatttgg	tcggtgaatt	tttaaaataa	catttttcaag	ataattttta	gttaatcaaa	1260
acattccacg	cattttctagt	ttcccaaatt	tctctaaatt	tcaggtgg	ctagcaacta	1320
acattgccag	cacaatggat	ctcgagggat	cttcataacc	taccagttct	gcgcctgcag	1380
gtcgcggccg	cgactctcta	gacgcgtaag	cttactagca	taacccttg	gggcctctaa	1440
acgggtcttg	aggggttttt	tgagcttctc	gccctatagt	gagtcgtatt	acagcttgag	1500
tattctatag	tgtcacctaa	atagcttggc	gtaatcatgg	tcatagctgt	ttcctgtgtg	1560
aaattgttat	ccgctcacaa	ttccacacaa	catacgagcc	ggaagcataa	agtgtaaagc	1620
ctgggggtgcc	taatgagtga	gctaactcac	attaattg	ttgcgctcac	tgcccgcctt	1680
ccagtcggga	aacctgtcgt	gccagctgca	ttaatgaatc	ggccaacgcg	cggggagagg	1740
cggtttgctg	attgggcgct	cttcgccttc	ctcgcctact	gactcgcctg	gctcggctcgt	1800
tcggctgcgg	cgagcggtat	cagctcactc	aaaggcggt	atacggttat	ccacagaatc	1860
aggggataac	gcaggaaaga	acatgtgagc	aaaaggccag	caaaaggcca	ggaaccgtaa	1920
aaaggccgcg	ttgctggcgt	ttttcgatag	gctccgcccc	cctgacgagc	atcacaaaaa	1980
tcgacgctca	agtcagaggt	ggcgaacccc	gacaggacta	taaagatacc	aggcgtttcc	2040
ccctggaagc	tccctcgtgc	gctctcctgt	tccgaccctg	ccgcttaccc	gatacctgtc	2100
cgcttttctc	ccttcgggaa	gcgtggcgct	ttctcatagc	tcacgctgta	ggtatctcag	2160
ttcgggtgtag	gtcgttcgct	ccaagctggg	ctgtgtgcac	gaaccccccg	ttcagcccga	2220
ccgctgcgcc	ttatccggta	actatcgtct	tgagtccaac	ccggtaaagac	acgacttatc	2280
gccactggca	gcagccactg	gtaacaggat	tagcagagcg	aggtatgtag	tcggtgtcac	2340
agagtctctg	aagtggtggc	ctaactacgg	ctacactaga	aggacagtat	ttggtatctg	2400
cgctctgctg	aagccagtta	ccttcggaaa	aagagttgg	agctcttgat	ccggcaaaaca	2460
aaccaccgct	ggtagcgg	gtttttttgt	ttgcaagcag	cagattacgc	gcagaaaaaa	2520
aggatctcaa	gaagatcctt	tgatcttttc	tacggggctc	gacgctcagt	ggaacgaaaa	2580
ctcacgttaa	gggatttttg	tcatgagatt	atcaaaaagg	atcttcacct	agatcctttt	2640
aaattaaaaa	tgaagtttta	aatcaatcta	aagtatatat	gagtaaaact	ggtctgacag	2700
ttaccaatgc	ttaatcagtg	aggcacctat	ctcagcgatc	tgtctatttc	gttcatccat	2760
agttgcctga	ctccccgtcg	tgtagataac	tacgatacgg	gagggccttac	catctggccc	2820
cagtgcctgca	atgataccgc	gagacccacg	ctcaccggct	ccagatttat	cagcaataaa	2880
ccagccagcc	ggaaggggcg	agcgcagaag	tggctcctgca	actttatccg	cctccatcca	2940
gtctattaat	tgttgccggg	aagctagagt	aagtagttcg	ccagttaata	gtttgcgcaa	3000
cgttggttggc	attgctacag	gcacgtgg	gtcacgctcg	tcgtttggta	tggttcat	3060
cagctccgg	tccaacgat	caaggcgagt	tacatgatcc	ccatggttgt	gcaaaaaagc	3120
ggttagcttc	ttcgttcctc	cgatcgttgt	cagaagtaag	ttggccgcag	ttgttatcact	3180
catggttatg	gcagcactgc	ataattctct	tactgtcatg	ccatccgtaa	gatgcttttc	3240
tgtgactgg	gagtactcaa	ccaagtcatt	ctgagaatac	cgcgcccggc	gaccgagttg	3300
ctcttgcccc	gcgtcaatac	gggataatag	tgtatgacat	agcagaactt	taaaagtgct	3360

catcattgga	aaacggttctt	cggggcgaaa	actctcaagg	atcttaccgc	tgttgagatc	3420
cagttcgaatg	taaccactc	gtgcacccaa	ctgatcttca	gcattcttta	ctttcaccag	3480
cgtttctggg	tgagcaaaaa	caggaaggca	aaatgccgca	aaaaaggga	taaggcgac	3540
acggaaatgt	tgaatactca	tactcttcct	ttttcaatat	tattgaagca	tttatcaggg	3600
ttattgtctc	atgagcggat	acatatattga	atgtatttag	aaaaataaac	aaataggggt	3660
tccgcgcaca	tttccccgaa	aagtgccacc	tgacgtctaa	gaaaccatta	ttatcatgac	3720
attaacctat	aaaaataggc	gtatcacgag	gccctttcgt	ctcgcgcgtt	tcggtgatga	3780
cggtgaaaac	ctctgacaca	tgcagctccc	ggagacggtc	acagcttgct	tgtaagcgga	3840
tgccgggagc	agacaagccc	gtcagggcgc	gtcagcgggt	gttggcgggt	gtcggggctg	3900
gcttaactat	gcggcatcag	agcagattgt	actgagagtg	caccatatgc	ggtgtgaaat	3960
accgcacaga	tgcgtaagga	gaaaataccg	catcaggcga	aattgtaaac	gttaatatatt	4020
tgtaaaaatt	cgcggttaaat	atthgttaaa	tcagctcatt	ttttaaccaa	taggccgaaa	4080
tcggcaaaat	cccttataaa	tcaaaagaat	agaccgagat	agggttgagt	gthgttccag	4140
tttggaacaa	gagtcacta	ttaaagaacg	tggactccaa	cgtcaaaggg	cgaaaaaccg	4200
tctatcaggg	cgatggccca	ctacgtgaac	catcacccaa	atcaagttht	ttgcggtcga	4260
ggtgcccgtaa	agctctaaat	cggaacccta	aaggagccc	ccgatttaga	gcttgacggg	4320
gaaagccggc	gaacgtggcg	agaaaggaag	ggaagaaagc	gaaaggagcg	ggcgctaggg	4380
cgctggcaag	tgtagcggtc	acgctgcgcg	taaccaccac	acccgccgcg	cttaatgcgc	4440
cgctacaggg	cgcgctcatt	cgccattcag	gctgcgcaac	tgttggaag	ggcgatcggg	4500
gcgggcctct	tcgctattac	gccagctggc	gaaaggggga	tgtgctgcaa	ggcgattaag	4560
ttgggtaacg	ccagggttht	cccagtcacg	acgttgtaaa	acgacggcca	gtgaattgta	4620
atacgactca	ctatagggcg	aattcaaaaa	acccctcaag	acccgtttag	aggccccaag	4680
gggttatgct	agtgaattct	gcagggtacc	cggggatcct	ctagagatcc	ctcgacctcg	4740
agatccattg	tgctgg					4756

<210> 4

<211> 4643

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Plasmid pGX52

<400> 4

gagtgacca	tatgcggtgt	gaaataccgc	acagatgcgt	aaggagaaaa	taccgcatca	60
ggcgaaattg	taaacgttaa	tattttgtta	aaattcgcgt	taaatatttg	ttaaatcagc	120
tcatttttta	accaataggc	cgaaatcggc	aaaatccctt	ataaatcaaa	agaatagacc	180
gagatagggg	tgagtgttgt	tccagtttgg	aacaagagtc	cactatttaa	gaacgtggac	240
tccaacgtca	aaggggcgaa	aaccgtctat	cagggcgatg	gcccactacg	tgaaccatca	300
cccaaataca	gttttttgcg	gtcgaggtgc	cgtaaagctc	taaatcggaa	ccctaaaggg	360
agcccccgat	ttagagcttg	acggggaaaag	ccggcgaaag	tggcgagaaa	ggaagggaag	420
aaagcgaaag	gagcgggcgc	tagggcgctg	gcaagtgtag	cggtcacgct	cgcggttaacc	480
accacacccg	ccgcgcttaa	tgcgccgcta	cagggcgctg	ccattcgcca	ttcaggctgc	540
gcaactgttg	ggaaggcgga	tcggtgcggg	cctcttcgct	attacgccag	ctggcgaaaag	600
ggggatgtgc	tgcaaggcga	ttaagttggg	taacgccagg	gttttcccag	tcacgacgtt	660
gtaaaacgac	ggccagtga	ttgtataacg	actcactata	gggcgaattc	aaaaaacccc	720
tcaagacccg	tttagaggcc	ccaaggggtt	atgctagtga	attctgcagg	gtacccgggg	780
atcctctaga	gatccctcga	cctcgagatc	cattgtgctg	gcagccgatc	tccgtcttgt	840
gaagatctac	tccaccacca	tccgtatcga	tcagtccatc	ctcaccggag	aatctgtgtc	900
tgttatcaag	cacaccgact	ctgtgcgaga	tccacgcgct	gttaaccagg	acaagaagaa	960
ttgtctgttc	tcgggaacca	atgtcgcata	tggaaaggct	cgtggaatcg	tcttcggaac	1020
cggattgacc	actgaaatcg	gaaagatccg	taccgaaatg	gctgagaccg	agaatgagaa	1080
gacaccactt	caacagaagt	tggaagcaat	cggagagcaa	ctttccaagg	ttatctctgt	1140
tatttgcgtt	gctgtttggg	ctatcaacat	tggaacattc	aacgatccag	ctcacggttg	1200
atcatgggtt	aaggggagcaa	tctactactt	caaaatcgcc	gthgtctctg	ccgtcgtctg	1260
tattccagaa	ggacttccag	ctgtcatcac	cacgtgcctt	gccctcggaa	ctcgccgat	1320
ggccaagaag	aacgctattg	taagatccct	tccatccgct	gaaactcttg	gatgcacatc	1380
tgttatctgc	tctgacaaga	ctggaactct	caccaccaac	cagatgtctg	tgtcaaatat	1440

gttcacgcgt	ggacaagctt	ctggagacaa	catcaacttc	accgagttcg	ccatctccgg	1500
atccacctac	gagccagtcg	gaaaggtttc	caccaatgga	cgtgaaatca	accagctgc	1560
tggagaattc	gaatcactca	ccgagttggc	catgatctgc	gctatgtgca	atgattcatc	1620
tggtgattac	aatgagacca	agaagatcta	cgagaaagtc	ggagaagcca	ctgaaactgc	1680
tcttatcggt	cttgctgaga	agatgaatgt	tttcggaacc	tcgaaagccg	gactttcacc	1740
aaaggagctc	ggaggagttt	gcaaccgtgt	catccaacaa	aaatggaaga	aggagttcac	1800
actcgagttc	tcccgtgatc	gtaaatccat	gtccgcctac	tgcttcccag	cttccggagg	1860
atctggagcc	aagatgttcg	tgaaggaggc	cccagaagga	gttctcggaa	gatgcacca	1920
cgtcagagtt	aacggacaaa	aggttccact	cacctctgcc	atgactcaga	agattgttga	1980
ccaatgctgt	caatacggaa	ccggaagaga	tacccttcgt	tgtcttgccc	tcggccagca	2040
caatggatct	cgagggatct	tccataccta	ccagttctgc	gcctgcaggt	cgcgcccgcg	2100
actctctaga	cgcgtaagct	tactagcata	accccttggg	gcctctaaac	gggtcttgag	2160
gggttttttg	agcttctcgc	cctatagtga	gtcgtattac	agcttgagta	ttctatagt	2220
tcacctaaat	agcttggcgt	aatcatggtc	atagctgttt	cctgtgtgaa	attgttatcc	2280
gctcacaatt	ccacacaaca	tacgagccgg	aagcataaag	tgtaaagcct	ggggtgccta	2340
atgagtgagc	taactcacat	taattgcgtt	gcgtcactg	cccgctttcc	agtcgggaaa	2400
cctgtcgtgc	cagctgcatt	aatgaatcgg	ccaacgcgcg	gggagaggcg	gtttgcgtat	2460
tgggcgctct	tccgcttcct	cgctcactga	ctcgtcgcgc	tcggtcgctc	ggctgcggcg	2520
agcggtatca	gctcactcaa	aggcggtaat	acggttatcc	acagaatcag	gggataacgc	2580
aggaaagaac	atgtgagcaa	aaggccagca	aaaggccagg	aaccgtaaaa	aggccgcgtt	2640
gctggcggtt	ttcgataggc	tccgcccccc	tgacgagcat	cacaaaaaatc	gacgctcaag	2700
tcagaggtgg	cgaaacccga	caggactata	aagataccag	gcgtttcccc	ctggaagctc	2760
cctcgtgcgc	tctcctgttc	cgaccctgcc	gcttaccgga	tacctgtccg	cctttctccc	2820
ttcgggaagc	gtggcgcttt	ctcatagctc	acgctgtagg	tatctcagtt	cgggtgtagg	2880
cgttcgctcc	aagctgggct	gtgtgcacga	acccccggt	cagcccagac	gctgcgcctt	2940
atccggtaac	tatcgtcttg	agtccaaccc	ggtaagacac	gacttatcgc	cactggcagc	3000
agccactggt	aacaggatta	gcagagcgag	gtatgtaggc	ggtgctacag	agttcttgaa	3060
gtggtggcct	aactacggct	acactagaag	gacagtattt	ggtatctgcg	ctctgctgaa	3120
gccacttacc	ttcggaaaaa	gagttggtag	ctcttgatcc	ggcaaacaaa	ccaccgctgg	3180
tagcgggtgg	ttttttgttt	gcaagcagca	gattacgcgc	agaaaaaaag	gatctcaaga	3240
agatcctttg	atcttttcta	cggggtctga	cgctcagtg	aacgaaaact	cacgttaagg	3300
gatttttggt	atgagattat	caaaaaggat	cttcacctag	atccttttaa	attaaaaatg	3360
aagtttttaa	tcaatctaaa	gtatatatga	gtaaacttgg	tctgacagtt	accaatgctt	3420
aatcagtgag	gcacctatct	cagcgatctg	tctatttctg	tcattccatag	ttgcctgact	3480
ccccgtcgtg	tagataacta	cgatacggga	gggcttacca	tctggcccca	gtgctgcaat	3540
gataccgcga	gacccacgct	caccggctcc	agatttatca	gcaataaacc	agccagccgg	3600
aagggccgag	cgcagaagtg	gtcctgcaac	tttatccgcc	tccatccagt	ctattaattg	3660
ttgccgggaa	gctagagtaa	gtagtctgcc	agttaatatg	ttgcgcaacg	ttgttggcat	3720
tgctacaggc	atcgtggtgt	cacgctcgtc	gtttgggtatg	gcttcattca	gctccggttc	3780
ccaacgatca	aggcgagtta	catgatcccc	catgttggtg	aaaaaagcgg	ttagctcctt	3840
cggctcctccg	atcgttgtca	gaagtaagtt	ggccgcagtg	ttatcactca	tggttatggc	3900
agcactgcac	aattctctta	ctgtcatgcc	atccgtaaga	tgcttttctg	tgactggtga	3960
gtactcaacc	aagtcattct	gagaataccg	cgcccgcgca	ccgagttgct	cttgcccggc	4020
gtcaatacgg	gataatagt	tatgacatag	cagaacttta	aaagtgtcta	tcattggaaa	4080
acgttcttcg	gggcgaaaa	tctcaaggat	cttaccgctg	ttgagatcca	gttcgatgta	4140
acccactcgt	gcacccaact	gatcttcagc	atcttttact	ttcaccagcg	tttctgggtg	4200
agcaaaaaaca	ggaaggcaaa	atgccgcaaa	aaagggaata	agggcgacac	ggaaatgttg	4260
aatactcata	ctcttccttt	ttcaatatta	ttgaagcatt	tatcagggtt	attgtctcat	4320
gagcggatac	atatttgaat	gtatttagaa	aaataaacia	ataggggttc	cgcgcacatt	4380
tccccgaaaa	gtgccacctg	acgtctaaga	aaccattatt	atcatgacat	taacctataa	4440
aaataggcgt	atcacgaggc	cctttcgtct	cgcgcggttc	ggtgatgacg	gtgaaaacct	4500
ctgacacatg	cagctcccgg	agacggtcac	agcttgtctg	taagcggatg	ccgggagcag	4560
acaagcccgt	cagggcgctg	cagcgggtgt	tggcgggtgt	cggggctggc	ttaactatgc	4620
ggcatcagag	cagattgtac	tga				4643

<210> 5

<211> 4454

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Plasmid pGX104

<400> 5

gagtgcacca	tatgcggtgt	gaaataccgc	acagatgcgt	aaggagaaaa	taccgcatca	60
ggcgaaattg	taaacgttaa	tattttgtta	aaattcgcgt	taaatatttg	ttaaatacagc	120
tcattttttta	accaataggc	cgaaatcggc	aaaatccctt	ataaatcaaa	agaatagacc	180
gagatagggg	tgagtgttgt	tccagtttgg	aacaagagtc	cactatttaa	gaacgtggac	240
tccaacgtca	aagggcgaaa	aaccgtctat	cagggcgatg	gcccactacg	tgaaccatca	300
cccaaataca	gttttttgcg	gtcgaggtgc	cgtaaagctc	taaatcggaa	ccctaaaggg	360
agcccccgat	ttagagcttg	acggggaaa	ccggcgaaac	tggcgagaaa	ggaaggggag	420
aaagcgaaag	gagcgggcgc	tagggcgctg	gcaagtgtag	cggtcacgct	gcgcgtaacc	480
accacaccgc	ccgcgcttaa	tgcgcgcgta	cagggcgctg	ccattcgcca	ttcaggctgc	540
gcaactgttg	ggaagggcga	tcggtgcggg	cctcttcgct	attacgccag	ctggcgaaa	600
ggggatgtgc	tgcaaggcga	ttaagttggg	taacgccagg	gttttcccag	tcacgacgtt	660
gtaaaacgac	ggccagtga	ttgtaatacg	actcactata	gggcgaattc	aaaaaacccc	720
tcaagaccgc	tttagaggcc	ccaaggggtt	atgctagtga	attctgcagg	gtacccgggg	780
atcctctaga	gatccctcga	cctcgagatc	cattgtgctg	gaccgtggta	ctcttatgga	840
gctcgggaatc	tcgccaatcg	tcacttctgg	acttatcatg	caacttctcg	ccggagccaa	900
gatcatcgaa	gtcggagaca	caccaaagga	ccgtgctctt	ttcaacggag	cccagaaatg	960
taagccgaaa	agtgtgtgtt	ttcaatctct	aatttttgaa	cttttcagtg	ttcgggtatg	1020
tcatactgt	tggaacaagc	attgtctacg	tcattgtccg	actctacgga	gagccatcgg	1080
aaatcggagc	tggaatctgt	ctccttatcg	tcgtccaact	cgttatttgc	ggtctcatcg	1140
tcctccttct	cgacgagctt	ctccaaaagg	gatattgtct	cggatccgga	atttctctct	1200
tcattgccac	caacatctgt	gaaaccattg	tctggaaggc	attctccccg	gcaacaatga	1260
acaccggacg	tggaaccgag	ttcgaaggag	ccgtcattgc	tcttttccat	cttcttgcca	1320
cccgctccga	gaaggtccgt	gcccctcggt	aggctttcta	ccgtcaaaac	cttccaaact	1380
tgatgaactt	gatggctact	ttcctcggtt	ttgcggtggt	tatctacttc	caaggattcc	1440
gtgtcgacct	cccaatcaag	tctgcccgtc	accgtggaca	atacagcagc	taccaatca	1500
agctcttcta	cacctccaac	attccaatca	tccttcaatc	tgctctcgtc	tccaacctct	1560
acgttatctc	tcaggtttgt	tgcatctcag	tagtaccgtt	agatgtttat	ctttctctag	1620
agggctcaagt	tgcccgagaa	attttttgag	ttcattctca	agtctgatgg	aaaatgttta	1680
tttttcagat	gctcgccgga	aagttcggag	gaaacttctt	catcaacctt	ctcggtaacct	1740
ggtccgataa	caccggatac	agaagctacc	caactggagg	actctgctac	tatctttcac	1800
caccagagtc	tcttggacac	atcttcgaag	acccaatcca	ctgcaccagc	acaatggatc	1860
tcgagggatc	ttccatacct	accagttctg	cgcttcgagg	tcgcggccgc	gactctctag	1920
acgcgtaagc	ttactagcat	aaccctcttg	ggcctctaaa	cgggtccttg	ggggtttttt	1980
gagcttctcg	ccctatagtg	agtcgtatta	cagcttgagt	attctatagt	gtcacctaaa	2040
tagcttggcg	taatcatggt	catagctgtt	tcctgtgtga	aattgttatc	cgctcacaa	2100
ttcacacaac	atacagccgc	gaagcataaa	gtgtaaagcc	tgggggtgcct	aatgagtgag	2160
ctaactcaca	ttaattgcgt	tgcgctcact	gcccgccttc	cagtcgggaa	acctgtcgtg	2220
ccagctgcat	taatgaatcg	gccaacgcgc	ggggagaggc	ggtttgcgta	ttggggcgctc	2280
ttccgcttcc	tcgctcactg	actcgctgcg	ctcggctcgtt	cggctgcggc	gagcgggtatc	2340
agctcactca	aaggcggtaa	tacggttatc	cacagaatca	ggggataacg	caggaaagaa	2400
catgtgagca	aaaggccagc	aaaaggccag	gaaccgtaaa	aaggccgcgt	tgctggcggtt	2460
tttcgatagg	ctccgcccc	ctgacgagca	tcacaaaaat	cgacgctcaa	gtcagaggtg	2520
gcgaaacccg	acaggactat	aaagatacca	ggcgtttccc	cctggaagct	ccctcgtgcg	2580
ctctcctgtt	ccgaccctgc	cgcttaccgg	atacctgtcc	gcctttctcc	cttcggggaag	2640
cgtggcgctt	tctcatagct	cacgctgtag	gtatctcagt	tcggtgtagg	tcgttcgctc	2700
caagctgggc	tgtgtgcacg	aacccccctg	tcagcccagc	cgtgcgcct	tatccggtaa	2760
ctatcgtctt	gagttccaac	cggttaagaca	cgacttatcg	ccactggcag	cagccactgg	2820
taacaggatt	agcagagcga	ggtatgtagg	cggtgtctaca	gagttcttga	agtggtggcc	2880
taactacggc	tacactagaa	ggacagttat	tggtatctgc	gctctgctga	agccagttac	2940
cttcggaaaa	agagttggta	gctcttgatc	cggcaaacaa	accaccgctg	gtagcgggtg	3000
tttttttgtt	tgcaagcagc	agattacgcg	cagaaaaaaa	ggatctcaag	aagatccttt	3060
gatcttttct	acgggggtctg	acgctcagtg	gaacgaaaac	tcacgttaag	ggatttttgtt	3120

catgagatta	tcaaaaagga	tcttcaccta	gatacctttta	aattaaaaat	gaagtttttaa	3180
atcaatctaa	agtatatatg	agtaaacttg	gtctgacagt	taccaatgct	taatcagtga	3240
ggcacctatc	tcagcgatct	gtctatttcg	ttcatccata	gttgccctgac	tccccgctgt	3300
gtagataact	acgatacggg	agggtttacc	atctggcccc	agtgcctgcaa	tgataccgcg	3360
agaccacgc	tcaccggctc	cagattttatc	agcaataaac	cagccagccg	gaagggccga	3420
gcgcagaagt	ggtcctgcaa	ctttatccgc	ctccatccag	tctattaatt	gttgccggga	3480
agctagagta	agtagttcgc	cagttaatag	tttgcgcaac	gttggttgga	ttgctacagg	3540
catcgtgggtg	tcacgctcgt	cgtttggtat	ggcttcattc	agctccggtt	cccaacgatc	3600
aaggcgagtt	acatgatccc	ccatggttg	caaaaaagcg	gttagctcct	tcggtcctcc	3660
gatcgttggtc	agaagtaagt	tggccgcagt	gttatcactc	atgggttatgg	cagcactgca	3720
taattctctt	actgtcatgc	catccgtaag	atgcttttct	gtgactgggtg	agtactcaac	3780
caagtcattc	tgagaatacc	gcgcccggcg	accgagttgc	tcttgcccgg	cgtcaatacg	3840
ggataatagt	gtatgacata	gcagaacttt	aaaagtgtc	atcattggaa	aacgttcttc	3900
ggggcgaaaa	ctctcaagga	tcttaccgct	gttgagatcc	agttcgatgt	aaccactcgc	3960
tgaccccaac	tgatcttcag	catcttttac	tttcaccagc	gtttctgggt	gagcaaaaac	4020
aggaaggcaa	aatgccgcaa	aaaagggaat	aagggcgaca	cggaaatggt	gaatactcat	4080
actcttcctt	tttcaatatt	attgaagcat	ttatcagggt	tattgtctca	tgagcggata	4140
catatttgaa	tgtatttaga	aaaataaaca	aataggggtt	ccgcgcacat	ttccccgaaa	4200
agtgccacct	gacgtctaag	aaaccattat	tatcatgaca	ttaacctata	aaaataggcg	4260
tatcacgagg	ccctttcgtc	tcgcgcgttt	cgggtgatgac	ggtgaaaacc	tctgacacat	4320
gcagctcccg	gagacggtca	cagcttgtct	gtaagcggat	gccgggagca	gacaagcccg	4380
tcagggcgcg	tcagcgggtg	ttggcgggtg	tcggggctgg	cttaactatg	cggcatcaga	4440
gcagattgta	ctga					4454

<210> 6

<211> 4701

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Plasmid pGZ18

<400> 6

accagcttt	cttgatacaa	gtgggtgatct	ttccagcaca	atggatctcg	agggatcttc	60
catacctacc	agttctgctc	ctgcaggctg	cggccgcgac	tctctagacg	cgtaagctta	120
ctagcataac	cccttggggc	ctctaaacgg	gtcttgaggg	gttttttgag	cttctcgccc	180
tatagtgagt	cgtattacag	cttgagtatt	ctatagtgtc	acctaaatag	cttggcgtaa	240
tcatgggtcat	agctgtttcc	tgtgtgaaat	tggttatccgc	tcacaattcc	acacaacata	300
cgagccggaa	gcataaagtg	taaagcctgg	ggtgcctaata	gagtgaagta	actcacatta	360
attgcggttg	gctcactgcc	cgctttccag	tcgggaaacc	tgctcgtgcca	gctgcattaa	420
tgaatcggcc	aacgcgcggg	gagaggcggg	ttgcgtattg	ggcgtctctc	cgcttcctcg	480
ctcactgact	cgctgcgctc	ggctgttcgg	ctgcgcgcag	cggatcagc	tcactcaaa	540
gcggaataac	ggttatccac	agaatcaggg	gataacgcag	gaaagaacat	gtgagcaaaa	600
ggccagcaaa	aggccaggaa	ccgtaaaaag	gccgcgttgc	tggcgttttt	cgataggctc	660
cgccccctg	acgagcatca	caaaaatcga	cgctcaagtc	agaggtggcg	aaacccgaca	720
ggactataaa	gataccaggc	gtttccccct	ggaagctccc	tcgtgcgctc	tcctgttccg	780
accctgccgc	ttaccggata	cctgtccgcc	tttctccctt	cgggaagcgt	ggcgttttct	840
catagctcac	gctgtaggta	tctcagttcg	gtgtaggtcg	ttcgtctcaa	gctgggctgt	900
gtgcacgaac	ccccggttca	gcccgaaccg	tgccgcttat	cggtaacta	tcgtcttgag	960
tccaacccgg	taagacacga	cttatcgcca	ctggcagcag	ccactggtaa	caggattagc	1020
agagcgaggt	atgtaggcgg	tgctacagag	ttcttgaagt	ggtggcctaa	ctacggctac	1080
actagaagga	cagtatttgg	tatctgcgct	ctgctgaagc	cagttacctt	cggaaaaaga	1140
gttggttagct	cttgatccgg	caaacaaacc	accgctggta	gcggtgggtt	ttttgtttgc	1200
aagcagcaga	ttacgcgcag	aaaaaaagga	tctcaagaag	atcctttgat	cttttctacg	1260
gggtctgacg	ctcagtgga	cgaaaactca	cgttaaggga	ttttgggtcat	gagattatca	1320
aaaaggatct	tcacctagat	cctttttaa	taaaaatgaa	gtttttaa	aatctaag	1380
atatatgagt	aaacttggtc	tgacagttac	caatgcttaa	tcagtggagc	acctatctca	1440
gcgatctgtc	tatttcggtc	atccatagtt	gcctgactcc	ccgtcgtgta	gataactacg	1500

atacgggagg	gcttaccatc	tggccccagt	gctgcaatga	taccgcgaga	cccacgctca	1560
ccggctccag	atztatcagc	aataaaccag	ccagccggaa	gggcccagcg	cagaagtggg	1620
cctgcaactt	tatccgcctc	catccagtct	attaattgtt	gccgggaagc	tagagtaagt	1680
agttcgccag	ttaatagttt	gcgcaacgtt	gttggcattg	ctacaggcat	cgtggtgtca	1740
cgctcgctcg	ttggtatggc	ttcattcagc	tccggttccc	aacgatcaag	gcgagttaca	1800
tgatcccca	tgttgtgcaa	aaaagcgggt	agctccttcg	gtcctccgat	cgttgtcaga	1860
agtaagttgg	ccgcagtgtt	atcactcatg	gttatggcag	cactgcataa	ttctcttact	1920
gtcatgccat	ccgtaagatg	cttttctgtg	actggtgagt	actcaaccaa	gtcattctga	1980
gaataccgcg	cccggcgacc	gagttgctct	tgcccggcgt	caatacggga	taatagtgtg	2040
tgacatagca	gaactttaaa	agtgtctatc	attggaaaac	gttcttcggg	gcgaaaactc	2100
tcaaggatct	taccgctgtt	gagatccagt	tcgatgtaac	ccactcgtgc	acccaactga	2160
tcttcagcat	cttttacttt	caccagcgtt	tctgggtgag	caaaaacagg	aaggcaaaat	2220
gccgcaaaaa	aggggaataag	ggcgacacgg	aaatgttgaa	tactcatact	cttccttttt	2280
caatattatt	gaagcattta	tcaggggttat	tgtctcatga	gcggatacat	atttgaatgt	2340
atthagaaaa	ataaacaat	aggggttccg	cgcacatttc	cccgaagagt	gccacctgac	2400
gtctaagaaa	ccattattat	catgacatta	acctataaaa	ataggcgtat	cacgaggccc	2460
tttcgtctcg	cgcgtttcgg	tgatgacggt	gaaaacctct	gacacatgca	gctcccggag	2520
acggtcacag	cttgtctgta	agcggatgcc	gggagcagac	aagcccgtca	gggcgcgtca	2580
gcgggtgttg	gcgggtgtcg	gggctggctt	aactatgcgg	catcagagca	gattgtactg	2640
agagtgcacc	atatgcggtg	tgaaataccg	cacagatgcg	taaggagaaa	ataccgcctc	2700
aggcgaaatt	gtaaacgtta	atattttgtt	aaaattcgcg	ttaaatattt	gttaaatacag	2760
ctcatttttt	aaccaatagg	ccgaaatcgg	caaaatccct	tataaatcaa	aagaatagac	2820
cgagataggg	ttgagtgttg	ttccagtttg	gaacaagagt	ccactattaa	agaacgtgga	2880
ctccaacgtc	aaagggcgaa	aaaccgtcta	tcagggcgat	ggcccactac	gtgaaccatc	2940
acccaaatca	agttttttgc	ggtcgagggt	ccgtaaagct	ctaaatcgga	accctaaagg	3000
gagccccoga	tttagagctt	gacggggaaa	gccggcgaa	gtggcgagaa	aggaaaggga	3060
gaaagcgaaa	ggagcgggcg	ctagggcgct	ggcagtgta	gcggtcacgc	tgcgcgtaac	3120
caccacaccc	gccgcgctta	atgcgcgct	acagggcgcg	tccattcgcc	attcaggctg	3180
cgcaactggt	gggaaggcg	atcgggtgcg	gcctcttcgc	tattacgcca	gctggcgaaa	3240
gggggatgtg	ctgcaaggcg	attaagtgtg	gtaacgccag	ggttttccca	gtcacgacgt	3300
tgtaaaacga	cggccagtga	attgtaatac	gactcactat	agggcgaatt	caaaaaacc	3360
ctcaagaccc	gttttagagg	cccaaggggt	tatgctagt	aattctgcag	ggtaccgagg	3420
gatcctctag	agatccctcg	acctcgagat	ccattgtgct	ggaaagcctt	tgcagggtcg	3480
gcaagccacg	tttggtgggt	gcgaccatcc	tccaaaatca	acaagtttgt	acaaaaaagc	3540
aggctatgcc	aagtacatgt	cgattgcgta	cgcgttcgta	atggttgctg	tgttagtcgc	3600
taccagcagt	caaattgttc	tcgagagtgc	gtttttacat	tatcccttca	tcctgattac	3660
gacaattttc	agctgttctc	gtcctctacat	ctctcttcat	tgtcacaatg	gtcggaatct	3720
tcttctttgc	tgcatgtctt	catccaaaag	aattcacgaa	tattatccat	ggtgtcgtat	3780
tcttctctcat	gattccatct	acatatgtgt	tctcactttt	atattcgctc	atcaatctca	3840
acgttatcac	gtgggggaact	cgtgaagctg	tcgctaaggc	aacgggacaa	aagacgaaaa	3900
aagcgccat	ggaacaattt	atagacagag	tgattgatat	tgtgaaaaag	ggattcagat	3960
taatcagttg	tcggggagaag	aaggaaacatg	aagagagacg	agagaaaatg	gaaaagaaaa	4020
tgacagaaat	ggagctagcc	ttgagaagta	ttgaggttat	ctttaacttt	agaaatgtga	4080
aattaataat	ttatttttcag	agtgggtgcg	acgtgaagaa	aattctcgat	gcaacagagg	4140
agaaggagaa	acgtgaagaa	gaaactcaaa	ctgcagattt	tccgattgaa	gagaacgtag	4200
agaagactca	aaaagagatt	cagaaggcaa	accgttatgt	gtggatgaca	agtcatagct	4260
tgaaagtttg	tgaacgagga	aaactgaaaa	gtgcggaaaa	ggtttttctg	aacgagctca	4320
tcaatgcata	tctgaaaccg	atcaagacga	cgccagctga	aatgaaagcc	gtcgccgaag	4380
gattggcttc	tctacgaaat	cagattgctt	tcactattct	tctcgttaat	tctcttcttg	4440
ctcttgccat	cttttttgatt	cagaaacaca	aaaatgtgct	cagcatcaag	ttctcgccaa	4500
tcagtaagca	atattacctt	tatgggtcaat	tcaaaaaatt	tgtttttttt	ttctagaaaa	4560
cttccgatgg	acgaaaatga	atgagatgac	tggacaatac	gaggaaaccg	atgaaccatt	4620
aaaaatagat	ccacttgga	tgggaattgt	tgttttcctt	ctaattattc	tttttgttca	4680
aactctcgga	atgcttctcc	a				4701

<210> 7

<211> 25

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:
Oligonucleotide primer C04H5.6F

<400> 7

tgctcagaga gtttctcaac gaacc

25

<210> 8

<211> 25

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:Oligonucleotide
primer C04H5.6R

<400> 8

caatgttagt tgctaggacc acctg

25

<210> 9

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:Oligonucleotide
primer K11D9.2bF

<400> 9

cagccgatct ccgtcttgtg

20

<210> 10

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:Oligonucleotide
primer K11D9.2bR

<400> 10

ccgagggcaa gacaacgaag

20

<210> 11

<211> 24

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:Oligonucleotide
primer Y57G11C.15F

<400> 11

accgtggtac tcttatggag ctcg

24

<210> 12

<211> 20
<212> DNA
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:Oligonucleotide
primer Y57G11C.15R

<400> 12
tgcagtggat tgggtcttcg

20

<210> 13
<211> 52
<212> DNA
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:Oligonucleotide
primer T25G3.2F

<400> 13
ggggacaagt ttgtacaaaa aagcaggcta tgccaagtac atgtcgattg cg

52

<210> 14
<211> 52
<212> DNA
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:Oligonucleotide
primer T25G3.2R

<400> 14
ggggaccact ttgtacaaga aagctggggtt ggagaagcat tccgagagtt tg

52

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
5 July 2001 (05.07.2001)

PCT

(10) International Publication Number
WO 01/48183 A3

(51) International Patent Classification⁷: **C12N 15/10**,
9/22, C07K 14/435, C12N 15/66, 15/70, 1/00

(74) Agent: **BAYLISS, Geoffrey, Cyril**; Boulton Wade Tennant,
Verulam Gardens, 70 Gray's Inn Road, London WC1X
8BT (GB).

(21) International Application Number: PCT/EP00/13149

(22) International Filing Date:
22 December 2000 (22.12.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
9930691.2 24 December 1999 (24.12.1999) GB

(71) Applicant (*for all designated States except US*): **DEV-
GEN NV** [BE/BE]; Technologiepark 9, B-9052 Zwij-
naarde (BE).

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU,
AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ,
DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR,
HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR,
LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ,
NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM,
TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM,
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian
patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European
patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE,
IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF,
CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— with international search report

(88) Date of publication of the international search report:
6 December 2001

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(72) Inventors; and
(75) Inventors/Applicants (*for US only*): **PLAETINCK, Geert** [BE/BE]; Pontstraat 16, B-9820 Merelbeke (BE). **MORTIER, Katherine** [BE/BE]; Paddenhoek 20, B-9830 St.-Martens Latem (BE). **LISSSENS, Ann** [BE/BE]; Tiensesteenweg 137, B-3010 Kessel-Lo (BE). **BOGAERT, Thierry** [BE/BE]; Wolvendreef 26g, B-8500 Kortrijk (BE).

(54) Title: IMPROVEMENTS RELATING TO DOUBLE-STRANDED RNA INHIBITION

(57) Abstract: There are described ways of improving the efficiency of double stranded RNA inhibition as a method of inhibiting gene expression in nematode worms such as *C. elegans*. In particular, the invention relates to the finding that changes in the genetic background of *C. elegans* result in increased sensitivity to double-stranded RNA inhibition.

WO 01/48183 A3



INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 00/13149

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/10 C12N9/22 C07K14/435 C12N15/66 C12N15/70
C12N1/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

BIOSIS, EPO-Internal, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	TIMMONS L ET AL: "Specific interference by ingested dsRNA" NATURE, MACMILLAN JOURNALS LTD. LONDON, GB, vol. 395, no. 6705, 29 October 1998 (1998-10-29), page 854 XP002103601 ISSN: 0028-0836 the whole document	1-4, 12-21, 23-25, 28-31, 34-39
A	FIRE A ET AL: "Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans" NATURE, GB, MACMILLAN JOURNALS LTD. LONDON, vol. 391, 19 February 1998 (1998-02-19), pages 806-811, XP002095876 ISSN: 0028-0836 cited in the application the whole document	1-4



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

* Special categories of cited documents:

A document defining the general state of the art which is not considered to be of particular relevance

E earlier document but published on or after the international filing date

L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

O document referring to an oral disclosure, use, exhibition or other means

P document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

& document member of the same patent family

Date of the actual completion of the international search

5 July 2001

Date of mailing of the international search report

20/07/2001

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Mateo Rosell, A.M.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 00/13149

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>SHARP PHILLIP A: "RNAi and double-strand RNA." GENES & DEVELOPMENT, vol. 13, no. 2, 15 January 1999 (1999-01-15), pages 139-141, XP002171268 ISSN: 0890-9369 the whole document</p> <p>---</p>	1-4
A	<p>RAY C ET AL: "GUT-SPECIFIC AND DEVELOPMENTAL EXPRESSION OF A CAENORHABDITIS ELEGANS CYSTEINE PROTEASE GENE" MOLECULAR AND BIOCHEMICAL PARASITOLOGY,NL,ELSEVIER SCIENCE PUBLISHERS, AMSTERDAM, vol. 51, 1992, pages 239-249, XP000572340 ISSN: 0166-6851 abstract</p> <p>---</p>	2-4,8
A	<p>RAND J B ET AL: "GENETIC PHARMACOLOGY: INTERACTIONS BETWEEN DRUGS AND GENE PRODUCTS IN CAENORHABDITIS ELEGANS" METHODS IN CELL BIOLOGY,LONDON,GB, vol. 84, 1995, pages 187-204, XP000956211 page 190, paragraph 1 -page 194, paragraph 4</p> <p>---</p>	1-4,8
A	<p>AVERY LEON ET AL: "The Caenorhabditis elegans unc-31 gene affects multiple nervous system-controlled functions." GENETICS, vol. 134, no. 2, 1993, pages 455-464, XP001011453 ISSN: 0016-6731 the whole document</p> <p>---</p>	2-4,8
A	<p>TAGESSON C ET AL: "INFLUENCE OF SURFACE-ACTIVE FOOD ADDITIVES ON THE INTEGRITY AND PERMEABILITY OF RAT INTESTINAL MUCOSA" FOOD AND CHEMICAL TOXICOLOGY, vol. 22, no. 11, 1984, pages 861-864, XP001009621 ISSN: 0278-6915 the whole document</p> <p>---</p> <p style="text-align: center;">-/--</p>	8

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 00/13149

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,A	<p>LYON CHRISTOPHER J ET AL: "The C. elegans apoptotic nuclease NUC-1 is related in sequence and activity to mammalian DNase II." GENE (AMSTERDAM), vol. 252, no. 1-2, 11 July 2000 (2000-07-11), pages 147-154, XP001009494 ISSN: 0378-1119 abstract; figure 3 page 151, right-hand column, paragraph 2 -page 153, right-hand column, paragraph 2</p> <p>---</p>	6,7
P,X	<p>WO 00 01846 A (MORTIER KATHERINE ;DEVGEN NV (BE); BOGAERT THIERRY (BE); PLAETINCK) 13 January 2000 (2000-01-13) cited in the application</p> <p>page 8, line 9 -page 10, line 22 page 12, line 14 -page 23, line 2 figures 5,9; examples 1-4</p> <p>---</p>	1-4, 12-21, 23-25, 28-31, 34-39
P,X	<p>WO 00 63425 A (FEICHTINGER RICHARD ;BEGHYN MYRIAM (BE); DEVGEN NV (BE); BOGAERT T) 26 October 2000 (2000-10-26) abstract page 2, line 14-30 page 6, line 18-33 page 7, line 12 -page 9, line 28 page 12, line 20 -page 13, line 25; example 3</p> <p>---</p>	2-5,8,9, 29-33
T	<p>TIMMONS LISA ET AL: "Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in Caenorhabditis elegans." GENE (AMSTERDAM), vol. 263, no. 1-2, 2001, pages 103-112, XP001009512 ISSN: 0378-1119 the whole document</p> <p>-----</p>	1-4, 12-21, 23-26, 28-31, 34-39

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP 00/13149

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 0001846 A	13-01-2000	AU 4907999 A EP 1093526 A GB 2349885 A NO 20010019 A	24-01-2000 25-04-2001 15-11-2000 05-03-2001
WO 0063425 A	26-10-2000	AU 3984600 A GB 2351152 A	02-11-2000 20-12-2000